Atty. Dkt. No. 034827-2301

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Sun, et al.

Title: METHODS AND COMPOSITIONS FOR THE DETECTION OF MUCOLIPIDOSIS IV MUTATIONS

Appl. No.: 10/754,446

Filing Date: 1/9/2004

Examiner: Kapushoc, Stephen Thomas

Art Unit: 1634

Confirmation Number: 7990

CERTIFICATE OF EXPRESS MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service's "Express Mail Post Office To Addressee" service under 37 C.F.R. § 1.10 on the date indicated below and is addressed to: **Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

EV 727835453 US (Express Mail Label Number) May 11, 2007 (Date of Deposit)

Juliene P. Britt
(Printed Name)

(Signature)

APPEAL BRIEF TRANSMITTAL

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith is an amendment in the above-identified application.

[X] Appeal Brief (19 pages including 3 appendices, and 5 exhibits).

[X] Credit Card Payment Form.

[X] Return Postcard.

[X] The fee required for additional claims is calculated below:

	Claims As Amended		Previously Paid For		Extra Claims Present		Rate		Additional Claims Fee
Total Claims:	16	-	36	=	0	x	$50.00	=	$0.00
Independent Claims:	2	-	5	=	0	x	$200.00	=	$0.00
First presentation of any Multiple Dependent Claims:						+	$360.00	=	$0.00
							CLAIMS FEE TOTAL	=	$0.00

[ ] Applicant hereby petitions for an extension of time under 37 C.F.R. §1.136(a) for the total number of months checked below:

[X]	Appeal Brief Fee	$500.00
	FEE TOTAL:	$500.00
[X]	Small Entity Fees Apply (subtract ½ of above):	$0.00
	TOTAL FEE:	$500.00

If any extensions of time are needed for timely acceptance of papers submitted herewith, applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 50-0872.

Please direct all correspondence to the undersigned attorney or agent at the address indicated below.

Respectfully submitted,

Date 05/11/2007

By _____

FOLEY & LARDNER LLP
Customer Number: 30542
Telephone: (858) 847-6722
Facsimile: (858) 792-6773

Barry S. Wilson
Attorney for Applicant
Registration No. 39,431

05-14-07

AF/IRJ

PATENT
034827-2301

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Sun et al.
Title:	METHODS AND COMPOSITIONS FOR THE DETECTION OF MUCOLIPIDOSIS IV MUTATIONS
Appl. No.:	10/754,446
Filing Date:	January 9, 2004
Examiner:	Kapushoc, S.T.
Art Unit:	1634
Conf. No.:	7990

CERTIFICATE OF EXPRESS MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service's "Express Mail Post Office To Addressee" service under 37 C.F.R. § 1.10 on the date indicated below and is addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

EV 727835453 US (Express Mail Label Number) — May 11, 2007 (Date of Deposit)

Juliene P. Britt
(Printed Name)

(Signature)

APPEAL BRIEF

Mail Stop Appeal Brief - Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicants (hereinafter "Appellants") hereby appeals the Final Rejection of claims 19-34, which corresponds to all claims pending in the application. This Appeal Brief follows a Notice of Appeal which was filed on March 12, 2007 and is accompanied by the requisite fee set forth in 37 C.F.R. § 1.17(f). If this fee is incorrect or if any additional fees are due in this regard, please charge or credit our Deposit Account No. 50-0872 for the appropriate amount.

05/15/2007 TBESHAH1 00000004 10754446
01 FC:1402
500.00 OP

Table of Contents

Table of Authorities

CASES

RULES

Real Party in Interest

The real party in interest in this appeal is Quest Diagnostics Incorporated, which is the assignee of the present application.

Related Appeals and Interferences

None.

Status of Claims

Claims 1-18 and 35-36 have been cancelled.

Claims 19-34 are pending in the application.

Claims 19-34 are the subject of this appeal.

Status of Amendments

The last claim amendments were presented in Appellants' Response of August 10, 2006. The claim amendments contained therein have been entered, examined, and appealed herein. No other amendments or submissions are pending in the application.

Summary of Claimed Subject Matter

Mucolipidosis type IV (MLIV) is a lyposomal storage disorder that affects the nervous system.[1] MLIV is an inherited disease and the majority of MLIV patients never develop the ability to speak or walk and remain at a developmental age of 1-2 years.[2] It is known that MLIV

[1] Specification at ¶¶ 3-4 and 7.

[2] Id. at ¶ 6.

is caused by a mutation in the MCOLN1 gene which encodes the mucolipin-1 membrane protein.[3]

Two different MCOLN1 mutations have been identified; a deletion mutation and a polymorphism mutation. The deletion mutant is characterized by a 6.4 kb deletion that includes exons 1-7.[4] The polymorphism mutant (IVS 3-2 A>G) is characterized by an A-to-G transition which causes a splice site mutation in the acceptor site of Intron 3 (hereinafter referred to as "the point mutation").[5] Because the site of the point mutation is contained within the genomic region deleted in the deletion mutation, only three allelic variants exist: (i) wt (no deletion, no point mutation), (ii) A>G (no deletion, point mutation present), and (iii) del (deletion mutation). The claimed subject matter relates to real-time PCR methods for detecting these mutations.

Support for claim 19 is found in the specification at ¶ 14.

Support for claims 20-22 is found in the specification at ¶¶ 10-11.

Support for claim 25 is found in the specification at ¶ 15.

Support for claims 26-31 and 34 is found in the specification at ¶¶ 10-11 and 16.

Grounds for Rejection to be Reviewed on Appeal

1. Claims 19 and 23-24 stand finally rejected under 35 U.S.C. § 103 as allegedly obvious over Edelmann et al. (Am. J. Hum. Genet. 70: 1023-1027, 2002), as evidenced by GenBank AF287270 (2000), in view of Doll et al. (Anal. Biochem. 301: 328-332, 2002).

2. Claims 25, 28, and 32-34 stand finally rejected under 35 U.S.C. § 103 as allegedly obvious over Edelmann et al. (Am. J. Hum. Genet. 70: 1023-1027, 2002), as evidenced by GenBank AF287270 (2000), in view of Doll et al. (Anal. Biochem. 301: 328-332, 2002).

3. Claims 20-22, 26-27, and 29-31 stand finally rejected under 35 U.S.C. § 103 as allegedly obvious over Edelmann et al. (Am. J. Hum. Genet. 70: 1023-1027, 2002) in view of

---

[3] Id. at ¶ 5.

[4] Id. at ¶ 4.

[5] Id.

Doll et al. (Anal. Biochem. 301: 328-332, 2002), GenBank AF287270 (2000), and Buck et al. (Biotechniques 27: 528-536, 1999).

Argument

1. Rejection of claims 19 and 23-24 under 35 U.S.C. § 103 over Edelmann et al., as evidenced by GenBank AF287270, in view of Doll et al.

1.1. The legal standard for obviousness.

In order to make a *prima facie* case of obviousness, the Examiner must demonstrate that the prior art (i) teaches or suggests every claim limitation, (ii) provides a motivation to combine (or modify) the teachings of the selected references, and (iii) provides a reasonable expectation of success. In re Vaeck, 947 F.2d 488, 493, 20 USPQ2d 1438 (CAFC 1991); MPEP § 2143. This is the "TSM" test for obviousness which was recently affirmed by the Supreme Court. KSR Int'l Co. v. Teleflex Inc., No. 04-1350, 550 U.S. ____, slip op. at 15 (2007). In explicating the correct standard for this test, the KSR Court reaffirmed previous holdings that an invention "is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." KSR, slip op. at 14.; see also, In re Rouffet, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457 (Fed. Cir. 1998). Furthermore, the Court warned the factfinder to be aware of the distortion caused by hindsight bias and to be cautious of arguments reliant upon *ex post* reasoning. KSR, slip op. at 17.

1.2. Appellants' claimed invention.

Claim 19 provides methods for real-time detection the MLIV deletion mutation (i.e., the 6.4 kb deletion that includes exons 1-7 of the MCOLN1 gene). Oligonucleotide primers (e.g., SEQ ID NOs.: 3 and 4; "PRI primers") which bind to nucleotides 100-500 and 6956-7356 of the MCOLN1 gene (SEQ ID NO: 8) are used for amplification.[6] These flanking primers result in an amplicon from the deletion mutant that is, at most, 800 nucleotides in length, but may be shorter based on specific primer selection. The amplicon, if present, is detected using a polynucleotide

[6] Specification at ¶¶ 36 and 50.

probe (e.g., SEQ ID NO.: 7) which is labeled with a donor fluorophore and an acceptor fluorophore (i.e., a quencher).[7] When unbound, the donor and acceptor fluorophores are in close proximity so little or no fluorescent signal from the donor fluorophore is detected. Upon binding of the labeled probe to the PCR product, the donor and acceptor fluorophores become spatially separated and the fluorescent signal from the donor fluorophore is increased.[8] In one variation of the claimed method, the PCR reaction is performed in the presence of a polymerase with 5' exonuclease activity which cleaves the double-stranded product formed by the hybridization of the probe and the PCR product.[9] This ensures permanent separation of the donor and acceptor fluorophores resulting in a robust and long-lasting increase in detectable fluorescence from the donor fluorophore.

1.3. Edelmann et al. and Doll et al. fail to teach or suggest every element of the claimed method.

The combination of Edelmann et al. and Doll et al. fails to teach every element in Appellants' method of claim 19. Edelmann et al. disclose a multi-step method for detecting MCOLN1 gene mutations. Edelmann et al. first perform a multiplex PCR amplification that results in one amplicon from the deletion mutant, if present, and a second amplicon encompassing the site of the point mutation. The PCR product(s) was immobilized and probed with radio-labeled allele-specific oligonucleotides for the point mutation, its wildtype, and the deletion mutation.[10] Thus, as acknowledged by the Examiner, Edelmann et al. fail to disclose genotyping multiple mutations (including the deletion mutation) of the MCOLN1 gene using real-time PCR.[11]

Doll et al. does not provide what Edelmann et al. lack. Doll et al. provide a method for detecting single nucleotide polymorphisms (i.e., point mutations) using fluorogenically-labeled probes. But, Doll et al. do not teach or suggest a method for detecting deletion mutations, as required by claim 19. Doll et al. recognized the existence of deletion mutations in the gene of

---

[7] Id. at ¶¶ 11 and 16-18.

[8] Id. at ¶ 45.

[9] Id. at ¶¶ 46-47 and claims 24 and 33.

[10] Edelmann et al. at page 1024, right column, first paragraph.

[11] Final Office Action mailed October 11, 2006, at page 6, first paragraph.

interest (NAT1).[12] However, despite this recognition, Doll et al. do not present a method for their detection. Thus, the Examiner has purely and improperly relied on a hindsight analysis, using nothing more than Appellants' specification, to "adapt" the method of Doll et al. for the detection of a deletion mutation.

Neither Edelmann et al. nor Doll et al. teach or suggest a method for the real-time detection of a deletion mutation in the MCOLN1 gene as required by claim 19. Because the combination of prior art asserted by the Examiner fails to teach every element of claim 19, this rejection should be reversed and withdrawn.

1.4. There is no motivation to combine the teachings of Edelmann et al. and Doll et al.

The Examiner fails to demonstrate that the prior art provides a motivation to combine the methods of Edelmann et al. and Doll et al. in an attempt to derive the method of claim 19. Edelmann et al. are concerned with detecting two different types of mutations in the MCOLN1 gene; a point mutation and a deletion mutation. Edelmann et al. use a method in which the genomic regions of interest are amplified by PCR. The PCR product is then probed, in a second step, using radio-labeled allele-specific oligonucleotides.

There exists no motivation to combine the teachings of Doll et al. with Edelmann et al. Each of the mutations detected by Doll et al. are point mutations.[13] Doll et al. are not concerned with detecting deletion mutations despite the fact that Doll et al. recognize their existence as alternate NAT1 alleles (the gene of interest to Doll et al.).[14] The Examiner has failed to identify a reason why a skilled artisan, understanding the method of Edelman et al., would be motivated to modify the portion of that method which relates to detecting deletion mutations with anything taught by Doll et al. which is directed solely to the detection of point mutations.

In sum, the combination of Doll et al. and Edelmann et al. fail to teach or suggest the use of allele-specific oligonucleotides to detect deletion mutations, as required in claim 19. Furthermore, there exists no motivation to combine the methods of Doll et al. and Edelmann et al. because Doll et al. are concerned with mutations in a gene unrelated to the MCOLN1 gene

[12] Doll et al. at Table 1.

[13] Id. at Table 2 and page 331, first paragraph.

[14] Id. at Table 1.

and, the mutations detected by Doll et al. are point mutations only. Appellants' respectfully submit that the Examiner has failed to make a *prima facie* case of obviousness and request that this rejection be reversed and withdrawn.

2. Rejection of claims 25, 28 and 32-38 under 35 U.S.C. § 103 over Edelmann et al., as evidenced by GenBank AF287270, in view of Doll et al.

Claims 28 and 32-38 each depend from claim 25. The method of claim 25 may be used to simultaneously detect the deletion mutation and the point mutation of the MCOLN1 gene. The deletion mutation is detected using substantially the same method as described above and encompassed by claim 19. The point mutation is detected by the further addition of PCR primers (e.g., SEQ ID NOs.: 1 and 2) which flank the mutation site.[15] The presence of the point mutation is determined using fluorophore-labeled probe specific for that sequence (e.g., SEQ ID NO.: 6). Optionally, a fluorophore-labeled probe that is specific for the wildtype sequence (e.g., SEQ ID NO.: 5) may be used in addition to, or instead of the mutant-specific probe.

Appellants submit that, for the reasons provided above, the method of claim 19 is unobvious. The methods of claims 25, 28, and 32-38 encompass all of the limitations of claim 19 and are, therefore, also unobvious for the same reasons. Additional arguments for the patentability of claims 25, 28, and 32-38 follow.

2.1. Edelmann et al. and Doll et al. fail to teach or suggest every element of the claimed method.

Claim 25 encompasses a method for the real-time detection of the MCOLN1 deletion mutation and the point mutation. The method requires a multiplex PCR using a primer pair which flanks the deleted region and another primer pair which flanks the site of the point mutation. The two MCOLN1 mutations are necessarily represented on different amplicons and are detected in real-time using allele-specific oligonucleotides containing fluorescent labels.

For the reasons discussed in Section 1.3 (*supra*), Appellants reassert that the combination of Edelmann et al. and Doll et al. fail to teach every element in the method of claim 25 insofar as

[15] Specification at ¶¶ 13-14.

claim 25 encompasses the real-time detection of the deletion mutation of the MCOLN1 gene. Specifically, Edelmann et al. detect MCOLN1 mutations, including a deletion mutation, using a two-part method which cannot be used in a real-time PCR-based genotyping assay. Doll et al. do not remedy the deficiency of Edelmann et al. because Doll et al. do not detect any deletion mutation, let alone the simultaneous detection of a deletion mutation and a point mutation. Thus, the combination of Edelmann et al. and Doll et al. fail to teach every element of claim 25.

2.2. There is no reasonable expectation of success for combining the methods of Edelmann et al. and Doll et al.

Also missing from the combination of the methods of Edelmann et al. and Doll et al. is a teaching of how to simultaneously detect multiple mutations on multiple amplicons in real-time. The method of claim 25 results in the production of two distinct amplicons. The first amplicon, if present, encompasses the region of Intron 3 that contains the A>G transition site and is produced from the first and second oligonucleotide primers. The second amplicon, if present, is produced from the third and fourth oligonucleotide primers and is indicative of the presence of the MCOLN1 deletion mutant.

Edelmann et al. detect the multiple MCOLN1 gene mutations on multiple amplicons but, this method cannot be adapted for real-time detection. Edelmann et al. immobilize the amplification products on a solid support and then detect those products with a radio-labeled oligonucleotide probe.

This deficiency is not remedied by Doll et al. The method of Doll et al. detects multiple mutations on the a single amplicon. Doll et al. provide no guidance on how the method might be adapted for use with multiple PCR primers resulting in multiple amplicons. Thus, the combination Edelmann et al. and Doll et al. fails to teach, and provides no reasonable expectation that multiple types of mutations (i.e., point mutations and deletion mutations) can be simultaneously detected on multiple amplicons in a real-time PCR-based assay as recited in claim 25.

In sum, the rejection of claims 25, 28 and 32-38 is flawed in several respects. The combination of Edelmann et al. and Doll et al. fail to teach a method for the real-time detection of a deletion mutation in any gene and they specifically fail to teach a method for the real-time detection of the MCOLN1 deletion mutation. Furthermore, the combination of Edelmann et al.

and Doll et al. does not provide the artisan with a reasonable expectation of success because this combination does not demonstrate how to detect multiple type of mutations (deletion mutations and point mutations) on multiple amplicons in real-time. Therefore, Appellants respectfully submit that this rejection should be reversed and withdrawn.

3. Rejection of claims 20-22, 26-27 and 29-31 under 35 U.S.C. § 103 over Edelmann et al., in view of Doll et al., GenBank AF287270 and Buck et al.

Claims 20-22 depend from claim 19 and claims 26-27 and 29-31 depend from claim 25. Each of the reasons discussed above for the unobviousness of claims 19 and 25 are reasserted with respect to claim 20-22, 26-27 and 29-31. Appellants submit that those reasons alone or in combination are sufficient to prove these claims are also unobvious. Additional arguments for the patentability of claims 20-22, 26-27 and 29-31 follow.

3.1. The prior art does not teach the specific primers and probes recited in the rejected claims.

Claims 20-21 and claims 29-30 require the use of the oligonucleotide primers of SEQ ID NOs.: 3 and 4 for amplification of the deletion mutation of the MCOLN1 gene. Claims 26-27 require the use of oligonucleotide primers of SEQ ID NOs.: 1 and 2 for amplification of the point mutation of the MCOLN1 gene. Claims 23 and 31 require the use of oligonucleotide probe of SEQ ID NO.: 7 for detection of the deletion mutation of the MCOLN1 gene. The Examiner acknowledges that the prior art does not teach any of these specific oligonucleotide primers or probes.[16]

In an attempt to remedy this deficiency in the prior art, the Examiner points to GenBank AF287270 which provides the nucleic acid sequence of the MCOLN1 gene and that Buck et al. demonstrate the equivalence of primers. The Examiner alleges that this combination renders obvious the use of any primer based on any known nucleic acid sequence.[17] Appellants strongly disagree.

[16] Final Office Action mailed October 11, 2006 at page 12, second paragraph.

[17] Id. at page 13.

As an initial matter, with respect to claims 23 and 31 which relate to the use of the oligonucleotide probe of SEQ ID NO.: 7, Appellants point out that the Examiner has failed to provide any reasons why the use of this probe, which is not disclosed in the prior art, is obvious. Notwithstanding this oversight, the teachings of Buck et al. are not concerned with oligonucleotide probes and are, therefore, irrelevant to this rejection insofar as the rejection relates to the specific probes of claims 23 and 31.

Turning next to the issue of primers, Buck et al. demonstrate the equivalence of sequencing primers. Specifically, Buck et al. perform a sequencing reaction using every primer spaced at a three nucleotide interval over a 300 nucleotide sequence and demonstrate that virtually every primer was successful. Appellants emphasize that Buck et al. are concerned with testing the effectiveness of sequencing primers which are used individually for a DNA polymerase reaction that is randomly terminated prior to the completion of a full copy of the target DNA. By contrast, PCR primers are used in a reaction that creates a full copy of the target DNA in which the primers are used in pairs to define the boundaries of the target DNA.

Appellants further urge that Buck et al. do not render obvious the use of any MCOLN1 primers in the present invention because Buck et al. are not concerned with real-time amplification assays. As attested by Dr. Sun, the presently clamed invention requires the use of PCR primers and hybridization probes in a real-time format (e.g., a Taq-man assay) which is more complex than standard assays.[18] The probes and primers must be capable of working together because the probes must bind to the amplicons, under the PCR conditions, as the amplicons are generated. Dr. Sun concludes that there is no expectation that any set of PCR primers, combined with any oligonucleotide hybridization probe will be successful in a real-time amplification assay.[19]

Thus, the sequencing primers of Doll et al. cannot be equated with PCR primers generally, or with multiplex primers used in a real-time amplification assay format as required by the rejected claims. Appellants respectfully submit that is rejection is traversed and should be withdrawn.

---

[18] Declaration of Weiman Sun, Ph.D. under 37 CFR § 1.132, filed August 10, 2006.

[19] Id.

Conclusion

For the reasons discussed above, Appellants respectfully submit that claims 19-34 are in condition for allowance, and respectfully request that the rejections be withdrawn or reversed, and that the claims be allowed to issue.

Respectfully submitted,

Date: 05/11/2007

By: Barry Wilson

FOLEY & LARDNER
P.O. Box 80278
San Diego, CA 92138-0278
(858) 847-6700 (Voice)
(858) 792-6773 (Fax)

Richard J. Warburg. Reg. No. 32,327
By Barry Wilson, Reg. No. 39,431
Attorneys for Applicants

Appendix A: Claims Appendix

1-18. (Cancelled)

19. (Previously Presented) A method of determining the presence of a Mucolipidosis IV deletion mutation sequence in a nucleic acid, comprising,

a) contacting the nucleic acid with:

i) a first oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 100-500 of the MCOLN1 gene (SEQ ID NO: 8),

ii) a second oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 6956-7356 of the MCOLN1 gene (SEQ ID NO: 8), and

iii) an oligonucleotide probe that comprises a sequence complementary to a 13-30 bp segment of a fragment that is amplified using the first and second primer, wherein said probe is labeled with a detectable label which comprises a donor fluorophore and a quencher moiety, wherein said quencher moiety is optionally an acceptor fluorophore; and

b) conducting amplification by temperature cycling and monitoring the accumulation of amplified nucleic in real time by detecting an increase in donor fluorophore fluorescence or a decrease in acceptor fluorophore fluorescence which indicates the presence of the Mucolipidosis IV mutant sequence in the nucleic acid.

20. (Original) The method of claim 19 wherein the first oligonucleotide primer comprises a sequence that consists essentially of 5'-CTT GCT CTG TTG CCC AGG CT -3' (SEQ ID NO. 3).

21. (Currently amended) The method of claim 19 wherein the second oligonucleotide primer comprises a sequence that consists essentially of 5'-CTC ACC GTG CTG GAA GAC ACT -3' (SEQ ID NO. 4).

22. (Previously Presented) The method of claim 19 wherein the probe comprises a sequence that consists essentially of 5'- AGACC CAG GCC CAC AT- 3' (SEQ ID NO: 7).

23. (Original) The method of claim 19 wherein the donor fluorophore is 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC), 6-carboxyfluorescein (FAM) or tetrachloro-6-carboxyfluorescein (TET).

24. (Original) The method of claim 19 wherein amplification by temperature cycling is with a DNA polymerase with 5' exonuclease activity and wherein binding of the probe to amplified nucleic acid results in degradation of the probe during DNA synthesis.

25. (Previously Presented) A method of detecting the presence of one or two Mucolipidosis IV mutant sequences in a nucleic acid, comprising,

a) contacting the nucleic acid with:
   i) a first oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 5124-5524 of the MCOLN1 gene (SEQ ID NO: 8),
   ii) a second oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 5541-5941 of the MCOLN1 gene (SEQ ID NO: 8),
   iii) a first oligonucleotide probe that comprises a sequence complementary to a 13-30 bp segment of a fragment that is amplified using the first and second oligonucleotide primers, wherein said first oligonucleotide probe includes position 5534 of the MCOLN1 gene (SEQ ID NO: 8), wherein said first oligonucleotide probe is labeled with a first detectable label which comprises a donor fluorophore and a quencher moiety, wherein said quencher moiety is optionally an acceptor fluorophore:
   iv) a third oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 100-500 of the MCOLN1 gene (SEQ ID NO: 8),
   v) a fourth oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 6956-7356 of the MCOLN1 gene (SEQ ID NO: 8), and

vi) a second oligonucleotide probe that comprises a sequence complementary to a 13-30 bp segment of a fragment that is amplified using the third and fourth primers, wherein said probe wherein said second oligonucleotide probe is labeled with a second detectable label which comprises a donor fluorophore and a quencher moiety, wherein said quencher moiety is optionally an acceptor fluorophore, and wherein said second detectable label is distinguishable from said first detectable label; and

b) conducting amplification by temperature cycling and monitoring the accumulation of amplified nucleic in real time by detecting an increase in donor fluorophore fluorescence or an increase or decrease in acceptor fluorophore fluorescence, which indicates the presence of one or both of the Mucolipidosis IV mutant sequences in the nucleic acid, wherein said first and second primer and first probe detect a single base transition mutation and said third and fourth primer and second probe detect a deletion mutation.

26. (Original) The method of claim 25 wherein the first oligonucleotide primer comprises a sequence that consists essentially of 5'-AGC GGG CCG GAC TCA-3' (SEQ ID NO. 1).

27. (Original) The method of claim 25 wherein the second oligonucleotide primer comprises a sequence that consists essentially of 5'-TAA CCA CCA TCG GAT CAA TGT C-3' (SEQ ID NO. 2).

28. (Original) The method of claim 25 wherein the first probe comprises a sequence that consists essentially of 5'- CTGC CCA CGG TAC CT -3' (SEQ ID NO: 6).

29. (Original) The method of claim 25 wherein the third oligonucleotide primer comprises a sequence that consists essentially of 5'-CTT GCT CTG TTG CCC AGG CT -3' (SEQ ID NO. 3).

30. (Original) The method of claim 25 wherein the fourth oligonucleotide primer comprises a sequence that consists essentially of 5'-CTC ACC GTG CTG GAA GAC ACT -3' (SEQ ID NO. 4).

31. (Original) The method of claim 25 wherein the second probe comprises a sequence that consists essentially of 5'- AGACC CAG GCC CAC AT- 3' (SEQ ID NO: 7).

32. (Original) The method of claim 25 wherein the first or second donor fluorophore is 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC), 6-carboxyfluorescein (FAM) or tetrachloro-6-carboxyfluorescein (TET).

33. (Original) The method of claim 25 wherein amplification by temperature cycling is with a DNA polymerase with 5' exonuclease activity and wherein binding of the probe to amplified nucleic acid results in degradation of the probe during DNA synthesis

34. (Original) The method of claim 25 wherein said nucleic acid containing sample is also contacted with a third oligonucleotide probe comprising a sequence consisting essentially of 5'- TCTG CCC ACA GTA CCT -3' (SEQ ID NO: 5) that hybridizes to a wildtype sequence, wherein said third probe is labeled with a detectable label comprising a donor fluorophore and a quencher moiety wherein said quencher moiety is optionally an acceptor fluorophore, and wherein said third detectable label is distinguishable from said first and second detectable labels.

35-36. (Cancelled)

Appendix B: Evidence Appendix

1. Buck et al. Biotechniques 27: 528-536, 1999.

2. Doll et al. Anal. Biochem. 301: 328-332, 2002.

3. Edelmann et al., Am. J. Hum. Genet. 70: 1023-1027, 2002.

4. GenBank AF287270, 2000.

5. Declaration of Weiman Sun, Ph.D. Under 37 CFR § 1.132.

Appendix C: Related Proceedings Appendix

None.

Research Report

Design Strategies and Performance of Custom DNA Sequencing Primers

BioTechniques 27:528-536 (September 1999)

G.A. Buck[1], J.W. Fox[2], M. Gunthorpe[3], K.M. Hager[4], C.W. Naeve[5], R.T. Pon[6], P.S. Adams[7] and J. Rush[8]

[1]Virginia Commonwealth University, Richmond, VA; [2]University of Virginia, Charlottesville, VA; [3]University of California, San Francisco, CA; [4]Yale University, New Haven, CT; [5]St. Jude Children's Research Hospital, Memphis, TN, USA; [6]University of Calgary, Calgary, AB, Canada; [7]Trudeau Institute, Saranac Lake, NY and [8]Harvard Medical School, Boston, MA, USA

ABSTRACT

This study surveyed strategies of sequencing primer selection and evaluated primer performance in automated DNA sequencing. We asked participants to relate their preferred primer design strategies to identify primer characteristics that are considered most important in sequencing primer design. The participants preferred primers of 18–24 nucleotides (nt), 39%–58% G+C, a melting temperature (T_m) of 53°–65°C with a 1–2 nt 3' GC clamp, hairpin stems of less than 2–3 bp, homopolymeric runs of less than 4–5 nt, primer dimers of less than 3–4 bp and secondary priming sites of less than 3–4 bp. We provided a 300-bp test sequence and asked participants to submit sequences of 1–3 optimal sequencing primers. Submitted primers ranged from 17–24 nt and largely conformed to the preferred parameters. Submitted primers were distributed across the test sequence, although some sites were disfavored. Surprisingly, approximately 45% of the primers were selected "manually", more than by any software package. Each of 69 submitted and 95 control primers, distributed at 3-bp intervals across the test sequence, were synthesized, purified and tested using a Model 377 PRISM™ DNA Sequencer with dichlororhodamine dye terminator reagents (dRhodamine dye terminators). Approximately half of the control primers were also tested using rhodamine dye terminator reagents ("old" rhodamine dye terminators). The results indicated that primer physico-chemical characteristics thought to have a strong impact on sequencing performance had surprisingly little effect. Thus, primers with high or low percent G+C or T_m, strong secondary priming scores or long 3' homopolymeric stretches yielded excellent sequences with the dRhodamine dye terminator reagents, although these characteristics had a stronger effect when the old rhodamine reagents were used. The old rhodamine reagents gave sequences with a similar average read length, but the number of errors and ambiguities or "N's" was consistently higher. Moreover, the effects of the primer physico-chemical characteristics were also more evident with the old rhodamine dyes. We conclude that under optimal sequencing conditions with highly pure template and primer, many of the commonly applied primer design parameters are dispensable, particularly when using one of the new generation of sequencing reagents such as the dichlororhodamine dye terminators.

INTRODUCTION

The Nucleic Acids Research Committee (NARC) of the Association of Biomolecular Resource Facilities (ABRF) evaluates the procedures and performance of research facilities that perform nucleic acid synthesis and sequencing in academic, industrial and other institutions (5,6). These studies provide a measure of the industry state-of-the-art, as well as the level of performance, that can be expected from such facilities. Previous projects have examined general operations of DNA core laboratories (15), the accuracy of automated DNA sequencing in these facilities (12), and the performance of unpurified synthetic oligonucleotides as primers for automated DNA sequence analysis (14). The DNA Sequencing Research Committee of the ABRF has performed studies evaluating the ability of DNA core facilities to sequence difficult templates (2) and the performance of various sequencing strategies and chemistries (1). Herein, we have studied primer selection strategies used by DNA sequencing facilities and empirically examined the quality of the sequence data provided by primers selected by these facilities.

Despite a long-standing conviction within the DNA sequencing community that careful primer design is essential to ensure high quality data (c.f., 11,14,17,18), there is a paucity of empirical studies supporting this view. A variety of reasonable "rules of thumb" (13) suggesting optimal ranges for primer length, percent G+C, melting

Table 1. Predicted Optimal and Observed Physico-Chemical Characteristics of Primers for Automated DNA Sequencing or PCR

	Sequencing Primers[a]		PCR Primers[b]		Submitted Primers[c]	
	Optimum[d]	Range[e]	Optimum[d]	Range[e]	Mean[f]	Range[g]
Length	18–24 nt	15–40 nt	18–29 nt	15–40 nt	19.7 nt	16–24 nt
T_m[h]	52°–66°C	40°–95°C	50°–69°C	40°–95°C	66°C	48°–91°C
Percent G+C of primer	40%–61%	30%–70%	40%–61%	30%–70%	53%	29%–82%
3′ GC clamp[i]	1–2 nt	0–5 nt	1–2 nt	0–2 nt	1.1 nt	0–4 nt
Primer dimer[j]	3–4 bp	0–7 bp	3–4 bp	0–7 bp	-5.4	-1.6–16.5
Hairpin stems[k]	2–3 bp	0–6 bp	2–3 bp	0–6 bp	1.1 bp	0–5 bp
Homopolymer runs[l]	4–5 nt	3–10 nt	4–5 nt	3–10 nt	3.0 nt	2–5 nt
Secondary priming[m]	3–4	0–10 bp	3–4 bp	0–10 bp	49[n]	0–266[n]

[a]Parameter values preferred by participants for primers to be used in sequencing.
[b]Parameter values preferred for primers to be used in PCR experiments.
[c]Parameter values of the primers submitted by participants.
[d]Optimum values for each parameter as selected by the participants.
[e]The range of acceptable values selected by the participants.
[f]The mean values for each parameter calculated from the submitted primers.
[g]The range of values for each parameter calculated from the submitted primers.
[h]Values submitted by participants for primers to be used for sequencing or PCR and nearest neighbor T_m of primers submitted by participants.
[i]The 3′ clamp refers to the number of G or C bases at the 3′ terminus of the primer.
[j]Largest number of contiguous complementary bases permitting dimerization of primer or the stability of strongest potential primer dimer (kcal) as measured by Oligo 5.0.
[k]Largest number of bases capable of forming a hairpin stem in the primer.
[l]Number of bases in longest homolopolymeric run in the primer.
[m]Number of bases permitted in most stable secondary priming site.
[n]The priming efficiency as calculated by Oligo 5.0 for test sequence only (submitted primers).

temperature (T_m) and composition have led to the development of primer design software packages that identify primers conforming to these criteria (4,16,18). However, the success of these strategies in selection of high-quality sequencing primers, to our knowledge, has not been extensively examined. In many sequencing projects, primer design and synthesis represent the most significant costs and consume the bulk of effort and time. Therefore, it is extremely important that these primers yield high-quality sequence data.

To examine this issue, an e-mail survey containing general questions about laboratory functions and specific questions concerning important characteristics for primer selection was distributed. A 300-bp test sequence was provided for which participants were asked to design sequencing primers. The submitted primers and a set of control primers spanning the 300-bp test sequence at 3-bp intervals were synthesized and used to sequence the test template on a Model 377 PRISM™ Automated DNA Sequencer (PE Biosystems, Foster City, CA, USA). Due to the results of this study, current primer design rules of thumb may be streamlined to facilitate more efficient primer selection for sequencing projects.

MATERIALS AND METHODS

The Survey

In December of 1996 and January of 1997, members of the ABRF were requested via e-mail distributions, the ABRF Electronic Bulletin Board and a standard mailing, to participate in a study of DNA sequencing primer design. The distribution provided a 300-bp "test sequence" (Figure 1) that was selected and previously shown to contain no segments that affect sequence ladder extension (data not shown). Participants were asked to use the prevailing technology in their laboratories to design and submit the sequences of up to three sequencing primers in the forward direction. Participants were requested to select the best primers irrespective of their positions on the test sequence. All responses were anonymously screened and coded by Virginia Commonwealth University (VCU) Health Sciences Computer Center personnel. Identifying data were removed from each document before being forwarded to the Committee for analysis. Several respondees submitted uninterpretable data. Where possible, these respondees were identi-

fied by Computer Center personnel, and new information was requested. Every effort was made to maintain the integrity of the data and the anonymity of the participants.

Control and Submitted Primers

A panel of 95 primers were synthesized as controls. These 18 nucleotide (nt) primers spanned the 300-bp test sequence with their 5′ termini located at 3-bp intervals (i.e., the first began at bp 1 of the test sequence, the second began at bp 4, etc.). All primers were analyzed with the Oligo™ Program 5.0 (NBI/Genovus, Plymouth, MN, USA) (16) and the number of bases, percent G+C, T_m (nearest neighbor method described in Reference 9), internal structure, secondary priming sites, etc., were recorded.

The 69 submitted and 95 control primers were synthesized on a Model 3948 DNA Synthesis and Purification System (PE Biosystems) at three different sites. Synthesis was performed at the 40 nmol scale using PE Biosystems reagents and the standard synthesis, cleavage and purification cycles. An aliquot of each sample was analyzed by polyacrylamide gel electrophoresis (PAGE) and by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS). PAGE was performed using precast minigels (Novex, San Diego, CA, USA) following the protocol specified by the manufacturer. Mass analysis was performed on a 200-pmol sample mixed with 10 mg/mL 3-hydroxypicolinic acid matrix (in 50% acetonitrile, 0.1% trifluoracetic acid) on a Voyager RP MALDI-TOF/MS instrument (PerSeptive Biosystems, Framingham, MA, USA). Criteria for passing quality control were: (*i*) a single major band on the gel; (*ii*) appropriate mobility on the gel compared to molecular weight standards; (*iii*) a synthesis yield >0.5 ODU (absorption of sample in 1 mL at 260 nm with 1 cm pathlength set up on the 3948 Synthesizer); and (*iv*) a single peak of the expected mass by MALDI-TOF/MS. Any oligonucleotides that failed to meet these criteria were resynthesized.

Test Template and Preparation

The plasmid template was preselected to contain a test sequence lacking obstacles to sequence extension (data not shown) and purified by double banding in CsCl-ethidium bromide isopycnic density gradients (10).

DNA Sequence Analysis

Each of the purified and quality-tested oligonucleotides were used as a primer for DNA sequence analysis using dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® FS reagents (PE Biosystems) under standard reaction conditions as described by the manufacturer at 20 µL total vol containing 300 ng of template and 5 pmol of primer. Sequencing reactions were run for 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C in a GeneAmp® PCR System 9700 DNA Thermal Cycler (PE Biosystems). Reactions were analyzed on Model 377 PRISM Automated DNA Sequencers using 5% Long Ranger™ (FMC BioProducts, Rockland, ME, USA) gels with Tris-Borate-EDTA buffer (TBE; pH 8.3). Approximately 30% of the control primers were rerun using the same dRhodamine dye terminator/Taq FS kit to ensure reproducibility, and approximately 50% of the control primers were rerun using the old rhodamine dye terminator/Taq FS kit (PE Biosystems). The dRhodamine or dichlororhodamine reagents are modifications of the original old rhodamine dyes that have narrower emission spectra, less spectral overlap and more even peak heights than the earlier dyes (19). Each 5% polyacrylamide sequencing gel was electrophoresed for approximately 8 h, and the data was directly exported for analysis using Sequencher™ (Gene Codes, Ann Arbor, MI, USA). Using this software, the raw data was trimmed to remove 5′ and 3′ ambiguous sequences, so that the first and last 25 nt contained no ambiguities. Each result was aligned with the known sequence, and we recorded: (*i*) the number of nt between the 3′ end of the primer and the beginning of the trimmed data; (*ii*) the number of nt in each sequence read; and (*iii*) the number of errors, including miscalls, insertions, deletions and ambiguities in each 100-bp window through the end of the sequence read.

```
  1   ATCCACACGG CGCTTTGACT CCCCTTCTGA AAAAAAAGAA CACGTTGATT
 51   CCCCTTTTGA TTTCCCACGA ATGCGAAGCA CAATTCACGC ACAGCCAACG
101   GCAGGGAGAG ACGATAAGGT AAAATACACA CAAGTGGTGG AATCACAAAA
151   GGACACGCAC AGCACGCAAA TAAACATTGC ACACGGCATG ACATTTTGCG
201   ACCCACACAC AATGTGTACA CTACAATTAT ATGAAAATCC CTCCCCCATT
251   CCGCGGGTGC GCCGCAAAGG GCCAAAAACC CAAATGATCC ACTTTATTAT
```

Figure 1. Test sequence. Shown is the 300-bp sequence distributed to participants for design of forward sequencing primers. Participants were asked to design primers in the forward direction.

RESULTS

Participant Profiles

Thirty-nine laboratories participated in the study, submitting 69 primer sequences. Of the participants, approximately 77% provide synthesis services and approximately 85% provide sequencing services, but only approximately 50% provide primer design or walking services, and less than one third offer template preparation. Laboratories performing these services averaged annually approximately 3700 syntheses, 7000 sequence runs, 425 primer designs, 424 template purifications and less than 200 primer walking projects. Charges for services averaged $1.17/nt for 40–50 nmol and $2.19/nt for 150–200 nmol syntheses of a 25-nt unpurified primer and $24/reaction for DNA sequence analysis ($9 for gel only). The average price for a primer walking sequencing project of a 2-kbp insert in a plasmid vector was approximately $500 or $0.25/bp. Charges for "external users", where available, averaged approximately 17%–50% higher than charges for institutional users. DNA synthesis and sequencing instrumentation was dominated by PE Biosystems; i.e., 77% of synthesizers and 92% of sequencers, but Beckman

Instruments, PerSeptive Biosystems and LI-COR were also represented. Only approximately 15% of the participants maintained robotic systems for template preparation or sequencing. Finally, when asked if commercial suppliers had an impact on services provided by the facilities, almost 80% of the laboratories responded affirmatively. Approximately 38% had dropped

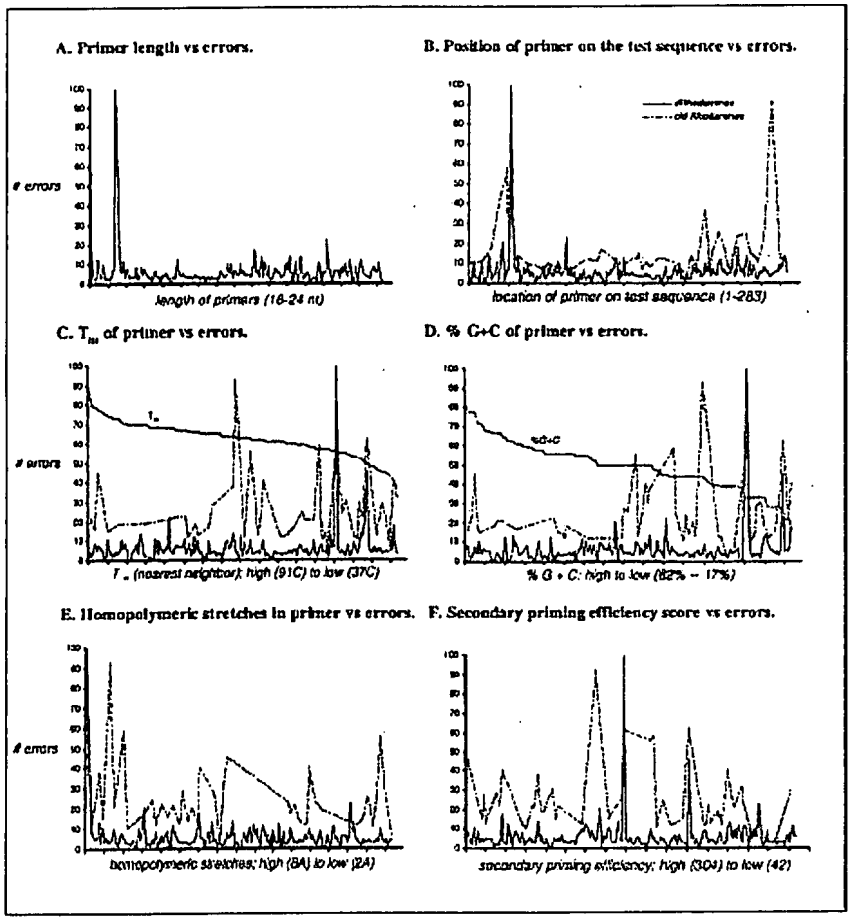

Figure 2. Primer characteristics vs. sequencing performance. The performance of sequencing primers, sorted according to various physico-chemical characteristics, was examined. The number of errors over the full-length read of each primer, determined as described in the Materials and Methods, was plotted against the results of each primer sorted according to the relevant parameter. (A) Primer length vs. errors. The primers were sorted according to their lengths in ascending order, and secondarily, according to their positions on the test sequence. The number of errors over the full-length read is plotted. Note that since all of the primers tested with the old rhodamine reagents were controls, and therefore 18-nt long, the data for old rhodamines are not included in this analysis. The X-axis distributes the primers from 16–24 nt in length; the y-axis indicates the number of errors over the full-length read of that primer. (B) Position of the primer on the test sequence. The primers were sorted according to the position of their 5′ nt on the test sequence (bp 1–283 on x-axis) and the number of errors in their full-length reads were plotted (y-axis). (C) T_m of the primer. The primers were sorted in descending order by their T_m's (91°–37°C on x-axis) and the number of errors in their full-length reads were plotted (y-axis). (D) The percent G+C of the primer. The primers were sorted in descending order (82%–17% on x-axis) by their percent G+C, and the number of errors in their full-length reads were plotted (y-axis). (E) Homopolymeric stretches in the primer. The primers were sorted according to first the number of nt (8–2 on x-axis) in the longest homopolymeric stretch, and secondarily according to the number of nt in the second longest homopolymeric stretch. The number of errors over the full-length read were plotted (y-axis). (F) Secondary priming efficiency of the primers. Each primer was examined for secondary priming sites in the vector and across the test sequence using Oligo 5.0. The priming efficiency score assigned by Oligo 5.0 was determined. The primers were sorted first according to the score of the strongest secondary priming site in the vector (304– 42 on x-axis) and secondarily according to the strongest secondary priming score in the test sequence. The number of errors across the full-length reads were plotted (y-axis).

ppm), due to the β effect. The same effect for compound **4** shifts the C-6 signal, which is observed at 66.2 ppm. Deprotection of HO-6 also affects the pattern of H-6,6′ in the ^{1}H NMR spectrum of **3**. In this case, the two protons appear as a doublet at the same chemical shift (4.07 ppm, Table 1).

Oxidation of HO-6 was performed with PCC as described (12) affording **5** (88%). Compound **5** showed a singlet at 9.2 ppm in the ^{1}H NMR spectrum, and a signal at δ 196.5 in the ^{13}C NMR spectrum, both diagnostic signals for the presence of the aldehyde group at C-6. For the reduction and debenzoylation steps, standard procedures were used. Compound [^{3}H]**1** showed the same chromatographic properties by TLC (Fig. 1, lane I) and HPAEC-PAD (Fig. 2A) than the nonradioactive compound. The specific activity (13 μCi/μmol) was calculated from the ratio between the radioactivity of the compound eluted from the HPAEC and the amount of material calculated by using mannose as standard.

Compound [^{3}H]**1** was used as substrate for the *exo*-β-D-galactofuranosidase from *P. fellutanum*, under the usual conditions (6). The enzymatic reaction could be followed by TLC (Fig. 1, lane II) and HPAEC-PAD analysis (Fig. 2B).

This is the first report on the synthesis of a radioactive substrate for β-D-galactofuranosidase. The radiolabeled material will facilitate detection of the enzyme in cultures or cellular fractions of microorganisms. In addition to being more sensitive, it can be used with colored biological materials without the interference caused in the colorimetric assay. The method could be useful for labeling other substrates in studies on the biosynthesis of galactofuranose glycans, like the galactan of *Mycobacterium tuberculosis* (13), although this chemical-labeling procedure would not be specific when other monosaccharides are present.

Acknowledgments. This work was supported by Agencia Nacional de Promoción Científica y Tecnológica, Argentina (ANPCYT, BID 802/OC-AR Pict 06-3036, and ANPCYT, BID 1201/OC-AR PICT 06-05133), and University of Buenos Aires.

REFERENCES

1. Lederkremer, R. M., and Colli, W. (1995) Galactofuranose-containing glycoconjugates in trypanosomatids. *Glycobiology* **5**, 547–552.
2. Rietschel-Berst, M., Jentoft, N. H., Rick, P. D., Pletcher, C., Fang, F., and Gander, J. E. (1977) Extracellular exo-β-galactofuranosidase from *Penicillium charlesii*. *J. Biol. Chem.* **252**, 3219–3226.
3. Daley, L. S., and Ströbel, G. A. (1983) β-D-Galactofuranosidase activity in *Helminthosporium sacchari* and its relationship to the production of helminthosporoside. *Plant Sci. Lett.* **30**, 145–154.
4. Cousin, M. A., Notermans, S., Hoogerhout, P., and Van Boom, J. H. (1989) Detection of β-galactofuranosidase production by *Penicillium* and *Aspergillus* species using 4-nitrophenyl β-D-galactofuranoside. *J. Appl. Bacteriol.* **66**, 311–317.
5. Miletti, L., Marino, C., Mariño, K., Lederkremer, R. M., Colli, W., and Manso Alves, M. J. (1999) Immobilized 4-aminophenyl 1-thio-β-D-galactofuranoside as a matrix for affinity purification of an exo-β-D-galactofuranosidase. *Carbohydr. Res.* **320**, 176–182.
6. Marino, C., Mariño, K., Miletti, L., Manso Alves, M. J., Colli, W., and Lederkremer, R. M. (1998) 1-Thio-β-D-galactofuranosides. Synthesis and evaluation as β-D-galactofuranosidase inhibitors. *Glycobiology* **8**, 901–904.
7. Varela, O., Marino, C., and Lederkremer, R. M. (1986) Synthesis of *p*-nitrophenyl β-D-galactofuranoside. A convenient substrate for β-D-galactofuranosidase. *Carbohydr. Res.* **155**, 247–251.
8. Nassau, P. M., Martin, S. L., Brown, R. E., Weston, A., Monsey, D., McNeil, M. R., and Duncan, K. (1996) Galactofuranose biosynthesis in *Escherichia coli* K-12: Identification and cloning of UDP-galactopyranose mutase. *J. Bacteriol.* **178**, 1047–1052.
9. Sanders, D. A. R., Staines, A. G., McMahon, S. A., McNeil, M. R., Whitfield, C., and Naismith, J. H. (2001) UDP-Galactopyranose mutase has a novel structure and mechanism. *Nature Struct. Biol.* **8**, 858–863.
10. Marino, C., Varela, O., and Lederkremer, R. M. (1989) Synthesis of galactofuranose disaccharides of biological significance. *Carbohydr. Res.* **190**, 65–76.
11. Randazzo, G., Capasso, R., Cicala, M. R., and Evidente, A. (1980) A simple method for detritylation of carbohydrate derivatives. *Carbohydr. Res.* **85**, 298–301.
12. Corey, E. J., and Suggs, J. W. (1975) Pyridinium chlorochromate. An efficient reagent for oxidation of primary and secondary alcohols to carbonyl compounds. *Tetrahedron Lett.* **31**, 2647–2650.
13. Kremer, L., Dover, L. G., Morehouse, C., Hitchin, P., Everett, M., Morris, H. R., Dell, A., Brennan, P. J., McNeil, M. R., Flaherty, C., Duncan, K., and Besra, G. S. (2001) Galactan biosynthesis in *Mycobacterium tuberculosis*. Identification of a bifunctional UDP-galactofuranosyltransferase. *J. Biol. Chem.* **276**, 26430–26440.

Rapid Genotype Method to Distinguish Frequent and/or Functional Polymorphisms in Human *N*-Acetyltransferase-1

Mark A. Doll and David W. Hein[1]

Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, Kentucky 40292

Received July 20, 2001; published online January 8, 2002

Human *N*-acetyltransferase 1 (NAT1)[2] catalyzes the *N*-acetylation of arylamine and hydrazine drugs and the O-acetylation of N-hydroxylated metabolites of ar-

[1] To whom correspondence should be addressed. Fax: (502) 852-7868. E-mail: d.hein@louisville.edu.

[2] Abbreviations used: NAT1, *N*-acetyltransferase 1; NAT2, *N*-acetyltransferase 2; PCR, polymerase chain reaction; FAM, 6-carboxyfluorescein; TET, tetrachloro-6-carboxyfluorescein; MGB, minor groove binder; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism; UTR, untranslated region.

Analytical Biochemistry **301**, 328–332 (2002)
doi:10.1006/abio.2001.5520
0003-2697/02 $35.00

TABLE 1

Frequency of *NAT1* Alleles in American and French Populations Determined by Comprehensive RFLP and SSCP Genotyping Methods

NAT1 allele	Nucleotide substitutions[a]	Number (%) identified in control populations			
		USA (Iowa)[b]	France[c]	Canada[d]	Germany[e]
*NAT1*4*	None (reference)	487 (74.2)	402 (74.4)	135 (70.3)	497 (72.4)
*NAT1*3*	**C^{1095}A**	20 (3.0)	15 (2.8)	0	21 (3.1)
*NAT1*10*	**T^{1088}A, C^{1095}A**	114 (17.4)	96 (17.8)	48 (25.0)	140 (20.4)
*NAT1*11* (*A* or *B*)	C^{-344}T, A^{-40}T, **G^{445}A**, G^{459}A, T^{640}G, 9-bp deletion 1065–1090, **C^{1095}A**	8 (1.2)	0	4 (2.1)	20 (2.9)
*NAT1*14* (*A* or *B*)	**G^{560}A, T^{1088}A, C^{1095}A**	13 (2.0)	20 (3.7)	5 (2.6)	4 (0.6)
*NAT1*15*	**C^{559}T**	2 (0.3)	1 (0.2)	0	0
*NAT1*17*	**C^{190}T**	7 (1.1)	3 (0.5)	0	1 (0.1)
*NAT1*19*	**C^{87}T**	0	0	0	0
*NAT1*22*	**A^{752}T**	5 (0.8)	0	0	3 (0.4)
*NAT1*26B*	TAA^{1091} insertion	0	1 (0.2)	0	0
*NAT1*28*	TAATAA$^{1085-90}$ deletion	0	1 (0.2)	0	0
*NAT1*29*	T^{1025} deletion, **T^{1088}A, C^{1095}A**	0	1 (0.2)	0	0
Total NAT1 alleles		456	540	192	686

[a] Nucleotide substitutions (compared with *NAT1*4* reference) detected in the new genotype method are in boldface.
[b] Reference (13).
[c] Reference (16).
[d] Reference (14).
[e] Reference (15).

TABLE 2

Primers and Fluorogenic Probes for SNP Determination[a]

97-Forward primer (58–85)	5′-gacttggaaacattaactgacattcttc-3′
97-Reverse primer (131–107) [74 bp]	5′-caatggatgttaaggttctcaaagg-3′
97C-TaqMan MGB probe (90–104)	FAM-ccagatc**C**gagctgt
97T-TaqMan MGB probe (90–105)	VIC-ccagatc**T**gagctgtt
190-Forward primer (143–165)	5′-tggacttaggcttagaggccatt-3
190-Reverse primer (220–198) [78 bp]	5′-gatgattgacctggagacaccat-3′
190C-TaqMan MGB probe (196–185)	FAM-caccc**C**atttc
190T-TaqMan MGB probe (195–183)	VIC-accc**A**atttctt
445-Forward primer (395–416)	5′-ggcagcctctggagttaatttc-3′
445-Reverse primer (506–481) [70 bp]	5′-tactgttcccttctgatttggtctag-3′
445G-TaqMan MGB probe (439–452)	FAM-ccttg**T**cttccg
445A-TaqMan MGB probe (437–454)	VIC-tgccttg**A**tcttccgtt
559/560-Forward primer (497–521)	5′-gggaacagtacattccaaatgaaga-3′
559/560-Reverse primer (595–571) [99 bp]	5′-ttgttcgaggcttaagagtaaagga-3′
559C/560G-TaqMan MGB probe (552–566)	FAM-caaatac**CG**aaaaat
559C/560A-TaqMan MGB probe (552–569)	VIC-caaatac**CA**aaaaatcta
559T/560G-TaqMan MGB probe (552–567)	TET-caaatac**TG**aaaaatc
752-Forward primer (713–734)	5′-ccctcacccataggagattcaa-3′
752-Reverse primer (793–765) [81 bp]	5′-tttctatttcttcctcactcagagtcttg-3′
752A-TaqMan MGB probe (743–762)	FAM-acaatacag**A**tctaatagag
752T-TaqMan MGB probe (743–762)	VIC-acaatacag**T**tctaatagag
1088/1095-Forward primer (1041–1066)	5′-gaaacataaccacaaacctttcaaa-3′
1088/1095-Reverse primer (1136–1112) [96 bp]	5′-aaatcaccaatttccaagataacca-3′
1088T/1095C-TaqMan MGB probe (1105–1079)	FAM-atctttaaaa**G**acattt**A**ttattatta
1088A/1095A-TaqMan MGB probe (1106–1079)	VIC-catctttaaaa**T**acattt**T**ttattatta
1088T/1095A-TaqMan MGB probe (1106–1078)	TET-catctttaaaa**T**acattt**A**ttattattat

[a] Nucleotide positions are indicated in parentheses. PCR product size is indicated in square brackets.

omatic and heterocyclic amines (1–10). NAT1 is encoded by a single 870-bp open reading frame that exhibits genetic polymorphism in human populations (11). Numerous studies have shown that the *NAT1* genetic polymorphism is associated with predisposition to various cancers from arylamine and heterocyclic amine carcinogens (reviewed in 12). The frequency of *NAT1* alleles has been difficult to assess because, until recently, most *NAT1* genotype data in the literature were based on the original method designed to detect only four *NAT1* alleles (12). More recently, comprehensive analyses of *NAT1* polymorphisms have been conducted by restriction fragment length polymorphism (RFLP) analysis (13–15) and single-strand conformation polymorphism (SSCP) analysis (16). As shown in Table 1, the comprehensive RFLP genotype methods identified eight *NAT1* alleles in American, Canadian, and German populations and the comprehensive SSCP genotype method identified nine *NAT1* alleles in a French population. However, three of these *NAT1* alleles (*NAT1*26B, *28,* and **29*) were identified in only one French subject and were not identified in the other ethnic groups. Consistent with these findings, our laboratory recently completed automated DNA sequencing of more than 1300 *NAT1* alleles from a Polish population and more than 1000 *NAT1* alleles from a Dutch population. The *NAT1* alleles identified following DNA sequencing were *NAT1*3, *4, *10, *11, *14, *15, *17,* and **22* (unpublished data). *NAT1* alleles with nucleotide substitutions in the coding region were recently expressed in yeast and those that possessed the $C^{97}T$, $C^{190}T$, $C^{559}T$, $G^{560}A$, or $A^{752}T$ nucleotide polymorphisms exhibited reduced *N*- and *O*-acetyltransferase activities (10). Relationships between *NAT1* genotype and phenotype in humans also have been reported (6, 9, 14, 17–21). The $T^{1088}A$ and $C^{1095}A$ polymorphisms in the 3′-untranslated region (UTR) are frequent (Table 1) and may confer functional consequences (18, 20, 21). The $G^{445}A$ polymorphism also may have functional consequences (4) and is necessary to distinguish the *NAT1*11A* and *B* alleles. Since frequencies of *NAT1* nucleotide polymorphisms have been determined by comprehensive RFLP, SSCP, and DNA sequencing methods, and since functional effects of the nucleotide polymorphisms have been assessed, we designed a rapid genotyping method to distinguish among the frequent and/or functional nucleotide polymorphisms in *NAT1* analogous to a genotype method recently designed for *NAT2* (22).

Nucleotide-specific polymerase chain reaction (PCR) primers and fluorogenic probes were designed using Primer Express (Version 1.5, Applied Biosystems, Foster City, CA) (Table 2). The fluorogenic MGB probes were labeled with a reporter dye (either FAM or VIC or TET) specific for one of the possible nucleotides identified at eight polymorphisms in the *NAT1* coding region or 3′-UTR: $C^{97}T$ (R^{33}Stop), $C^{190}T$ ($R^{64}W$), $G^{445}A$ ($V^{149}I$), $C^{559}T$ (R^{187}Stop), $G^{560}A$ ($R^{187}Q$), $A^{752}T$ ($D^{251}V$), $T^{1088}A$ (3′UTR), $C^{1095}A$ (3′UTR). The fluorogenic probes for the 559/560 and the 1088/1095 are distinguished using a three-probe system that has both nucleotide polymorphisms in the same probe. This was necessary because the 559/560 and 1088/1095 are too close together to use the conventional two-probe TaqMan assay. The nucleotide-specific primers amplify a segment of the *NAT1* gene flanking the probes.

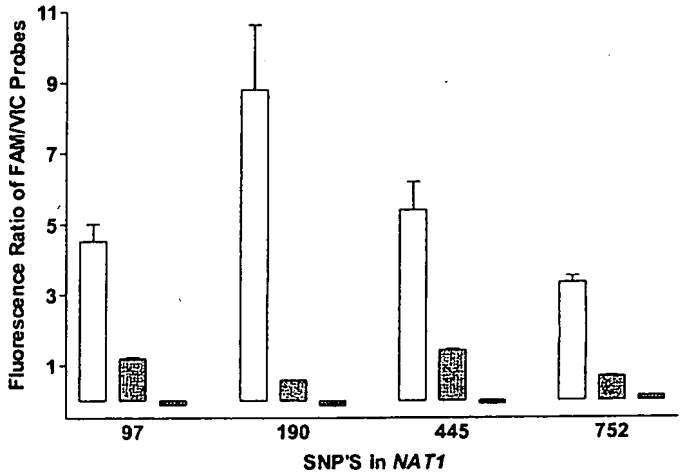

FIG. 1. Fluorescence ratio of FAM-labeled/VIC-labeled fluorogenic probes specific for the nucleotide polymorphisms in the *NAT1* coding region. Each bar represents the mean ± SE for determinations in DNA from three human subjects. Open bars represent DNA samples homozygous for 97C, 190C, 445G, and 752A, respectively. Solid bars represent DNA samples homozygous for 97T, 190T, 445A, and 752T, respectively. Crossed bars represent DNA samples heterozygous for each nucleotide polymorphism. The fluorescence ratio differed significantly ($P < 0.005$) among homozygous and heterozygous *NAT1* genotypes for each nucleotide polymorphism following one-way analysis of variance.

TaqMan Universal PCR Master Mix (Applied Biosystems) was used to prepare the PCR. The 2× mixture is optimized for TaqMan reactions and contains AmpliTaq Gold DNA polymerase, AmpErase, dNTPs with UTP, and a passive reference. Primers, probes, and DNA were added to final concentrations of 300 nM, 100 nM, and 0.5–2.5 ng/μl, respectively. Controls (no DNA template) were run to ensure that there was no amplification of contaminating DNA. The amplification reactions were carried out in an ABI Prism 7700 sequence detection system (Applied Biosystems) with two initial hold steps (50°C for 2 min, followed by 95°C for 10 min) and 40 cycles of a two-step PCR (92°C for 15 s, 60°C for 1 min). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the *NAT1* gene. The nucleotide present in the *NAT1* alleles was determined by the fluorescence ratio of the nucleotide-specific fluorogenic probes. The fluorescence signal increases when the

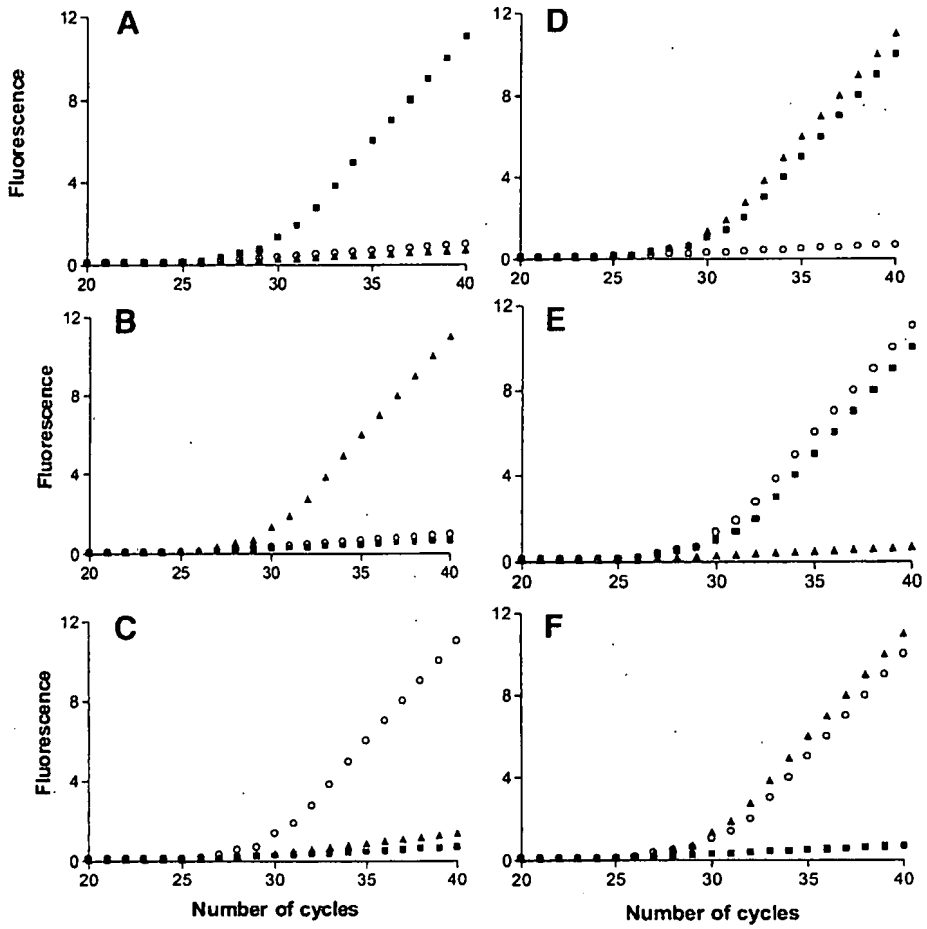

FIG. 2. Illustration of three-probe assay designed to distinguish 1088/1095 or 559/560 nucleotide combinations. The fluorescence signal is plotted as a function of cycle number. In each panel, squares represent signal from FAM-labeled probes, triangles represent signal from VIC-labeled probes, and circles represent signal from TET-labeled probes. (A) 1088TT/1095CC or 559CC/560GG; (B) 1088AA/1095AA or 559CC/560AA; (C) 1088TT/1095AA or 559TT/560GG; (D) 1088TA/1095CA or 559CC/560GA; (E) 1088TT/1095CA or 559CT/560GG; (F) 1088TA/1095AA or 559CT/560GA.

probe with the exact sequence match binds to the single-stranded template DNA and is digested by the 5′–3′-exonuclease activity of AmpliTaq Gold DNA polymerase (Applied Biosystems). Digestion of the probe releases the fluorescent reporter dye (either FAM or VIC or TET) from the quencher.

As shown in Figs. 1 and 2, the method readily distinguishes between the two possible nucleotides at each of the eight nucleotide polymorphisms identified in the *NAT1* coding and 3′-UTR: $C^{97}T$ (R^{33}Stop), $C^{190}T$ ($R^{64}W$), $G^{445}A$ ($V^{149}I$), $C^{559}T$ (R^{187}Stop), $G^{560}A$ ($R^{187}Q$), $A^{752}T$ ($D^{251}V$), $T^{1088}A$ (3′-UTR), $C^{1095}A$ (3′-UTR). Determination of the eight nucleotide polymorphisms showed complete concordance with a comprehensive RFLP method (13) and DNA sequencing. All *NAT1* alleles (*NAT1*3, NAT1*4, NAT1*10, NAT1*11A, NAT1*11B, NAT1*14A, NAT1*14B, NAT1*15, NAT1*17, NAT1*19, NAT1*22*) except for very rare ones can be detected with this method. Major advantages of this genotyping method are that it does not require post-PCR processing or the use of radioactivity. Since the method amplifies a much smaller segment of *NAT1* (74–99 bp) than is required for the comprehensive RFLP (1148 bp) or SSCP (1197 bp) genotyping methods (13, 16), it is much more effective in determining *NAT1* genotypes in human DNA samples derived from buccal cells or paraffin-embedded tissues. Since the new method does not require post-PCR processing, it is much faster and suitable for automated, high-throughput applications.

Acknowledgment. This work was partially supported by U.S. Public Health Service Grant CA-34627 from the National Cancer Institute.

REFERENCES

1. Minchin, R. F., Reeves, P. T., Teitel, C. H., McManus, M. E., Mojarrabi, B., Ilett, K. F., and Kadlubar, F. F. (1992) N- and O-acetylation of aromatic and heterocyclic amine carcinogens by

human monomorphic and polymorphic acetyltransferases expressed in COS-1 cells. *Biochem. Biophys. Res. Commun.* **185**, 839–844.

2. Hein, D. W., Doll, M. A., Rustan, T. D., Gray, K., Feng, Y., Ferguson, R. J., and Grant, D. M. (1993) Metabolic activation and deactivation of arylamine carcinogens by recombinant NAT1 and polymorphic NAT2 acetyltransferases. *Carcinogenesis* **14**, 1633–1638.
3. Hein, D. W., Rustan, T. D., Ferguson, R. J., Doll, M. A., and Gray, K. (1994) Metabolic activation of aromatic and heterocyclic *N*-hydroxyarylamines by wild-type and mutant recombinant human NAT1 and NAT2 acetyltransferases. *Arch. Toxicol.* **68**, 129–133.
4. Doll, M. A., Jiang, W., Deitz, A. C., Rustan, T. D., and Hein, D. W. (1997) Identification of novel allele at the human NAT1 acetyltransferase locus. *Biochem. Biophys. Res. Commun.* **233**, 584–591.
5. Butcher, N. J., Ilett, K. F., and Minchin, R. F. (1998) Functional polymorphism of the human arylamine *N*-acetyltransferase type-1 gene caused by C190T and G560A mutations. *Pharmacogenetics* **8**, 67–72.
6. Hughes, N. C., Janezic, S. A., McQueen, K. I., Jewett, M. A., Castranio, T., Bell, D. A., and Grant D. M. (1998) Identification and characterization of variant alleles of human acetyltransferase NAT1 with defective function using *p*-aminosalicylate as an *in vitro* and *in vivo* probe. *Pharmacogenetics* **8**, 55–66.
7. Lin, H. J., Probst-Hensch, N. M., Hughes, N. C., Sakamoto, G. T., Louie, A. D., Kau, I. H., Lin, B., K., Lee, D. B., Lin, J., Frankl, H. D., Lee, E. R., Hardy, S., Grant, D. M., and Haile, R. W. (1998) Variants of *N*-acetyltransferase NAT1 and a case–control study of colorectal adenomas. *Pharmacogenetics* **8**, 269–281.
8. Payton, M. A., and Sim, E. (1998) Genotyping human arylamine *N*-acetyltransferase type 1 (NAT1) variants. *Biochem. Pharmacol.* **55**, 839–844.
9. de Leon, J. H., Vatsis, K. P., and Weber, W. W. (2000) Characterization of naturally occurring and recombinant human *N*-acetyltransferase variants encoded by NAT1*. *Mol. Pharmacol.* **58**, 288–299.
10. Fretland, A. J., Doll, M. A., Leff, M. A., and Hein, D. W. (2001) Functional characterization of nucleotide polymorphisms in the coding region of *N*-acetyltransferase 1. *Pharmacogenetics* **11**, 511–520.
11. Vatsis, K. P., and Weber, W. W. (1993) Structural heterogeneity of Caucasian *N*-acetyltransferase at the NAT1 gene locus. *Arch. Biochem. Biophys.* **301**, 71–76.
12. Hein, D. W., Doll, M. A., Fretland, A. J., Leff, M. A., Webb, S. J., Xiao, G. H., Devanaboyina, U.-S., Nangju, N., and Feng, Y. (2000) Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. *Cancer Epidemiol. Biomarkers Prev.* **9**, 29–42.
13. Zheng, W., Deitz, A. C., Campbell, D. R., Wen, W.-Q., Cerhan, J. R., Sellers, T. A., Folsom, A. R., and Hein, D. W. (1999) *N*-Acetyltransferase genetic polymorphism, cigarette smoking, well-done meat intake, and breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **8**, 233–239.
14. Vaziri, S. A. J., Hughes, N. C., Sampson, H., Darlington, G., Jewett, M. A. S., and Grant, D. M. (2001) Variation in enzymes of arylamine procarcinogen biotransformation among bladder cancer patients and control subjects. *Pharmacogenetics* **11**, 7–20.
15. Cascorbi, I., Roots, I., and Brockmoller, J. (2001) Association of NAT1 and NAT2 polymorphisms to urinary bladder cancer: Significantly reduced risk in subjects with NAT1*10. *Cancer Res.* **61**, 5051–5056.
16. Lo-Guidice, J.-M., Allorge, D., Chevalier, D., Debuysere, H., Faxio, F., Lafitte, J.-J., and Broly, F. (2000) Molecular analysis of the *N*-acetyltransferase 1 gene (NAT1*) using polymerase chain reaction-restriction fragment-single strand conformation polymorphism assay. *Pharmacogenetics* **10**, 293–300.
17. Weber, W. W., and Vatsis, K. P. (1993) Individual variability in *p*-aminobenzoic acid N-acetylation by human *N*-acetyltransferase (NAT1) of peripheral blood. *Pharmacogenetics* **3**, 209–212.
18. Bell, D. A., Badawi, A. F., Lang, N. P., Ilett, K. F., Kadlubar, F. F., and Hirvonen, A. (1995) Polymorphism in the *N*-acetyltransferase 1 (NAT1) polyadenylation signal: Association of the NAT1*10 allele with higher N-acetylation activity in bladder and colon tissue. *Cancer Res.* **55**, 5226–5229.
19. Bruhn, C., Brockmoller, J., Cascorbi, I., Roots, I., and Borchert, H. H. (1999) Correlation between genotype and phenotype of the human arylamine *N*-acetyltransferase type 1. *Biochem. Pharmacol.* **58**, 5226–5229.
20. Yang, M., Katoh, T., Delongchamp, R., Ozawa, S., Kohsi, K., and Kawamoto, T. (2000) Relationship between NAT1 genotype and phenotype in a Japanese population. *Pharmacogenetics* **10**, 225–232.
21. Hein, D. W., McQueen, C. A., Grant, D. M., Goodfellow, G. H., Kadlubar, F. F., and Weber, W. W. (2000) Pharmacogenetics of the arylamine *N*-acetyltransferases: A symposium in honor of Wendell W. Weber. *Drug Metab. Dispos.* **28**, 1425–1432.
22. Doll, M. A., and Hein, D. W. (2001) Comprehensive human NAT2 genotype method using single nucleotide polymorphism-specific polymerase chain reaction primers and fluorogenic probes. *Anal. Biochem.* **288**, 106–108, doi:10.1006/abio.2000.4892.

Am. J. Hum. Genet. 70:1023–1027, 2002

Report

Carrier Screening for Mucolipidosis Type IV in the American Ashkenazi Jewish Population

Lisa Edelmann, Jianli Dong, Robert J. Desnick, and Ruth Kornreich

Department of Human Genetics, Mount Sinai School of Medicine, New York University, New York

Mutations in the *MCOLN1* gene cause mucolipidosis type IV (MLIV), a severely debilitating, autosomal recessive, lysosomal storage disorder. Approximately 80% of patients with MLIV are of Ashkenazi Jewish (AJ) descent, and two mutations, IVS3−2A→G and 511del6434, account for >95% of the mutant alleles in this population. To determine the carrier frequencies of these two mutations, 2,029 anonymous, unrelated, unaffected AJ individuals from the greater New York metropolitan area were screened. A multiplex PCR method coupled with allele-specific oligonucleotide hybridization was developed, to enable large-scale screening. The frequencies of the IVS3−2A→G and 511del6434 mutations were 0.54% and 0.25%, respectively, for a combined carrier frequency of 0.79%, or 1 in 127 individuals (95% CI 0.40%–1.17%). The addition of both AJ mutations causing this neurodegenerative disorder should be considered for prenatal carrier screening in this population.

Mucolipidosis type IV (MLIV [MIM 252650]) is a neurodegenerative lysosomal storage disorder that is inherited as an autosomal recessive trait. Clinically, the disease, which occurs primarily among Ashkenazi Jewish (AJ) individuals, is characterized by growth and psychomotor retardation, as well as ophthalmologic abnormalities, which include corneal clouding, progressive retinal degeneration, and strabismus (Berman et al. 1974; Riedel et al. 1985). There is clinical variability, but most patients never develop the ability to speak or walk and remain at a developmental level of age 1–2 years. Although the disorder is associated with the accumulation of cytoplasmic storage bodies, normal levels of lysosomal hydrolases are present, with no specific identifiable storage compound (Amir et al. 1987). Electron photomicrographs of tissue biopsies from patients with MLIV have shown a widespread heterogeneous accretion of lipids and water-soluble compounds, indicating that the etiology of the disease is probably not related to the deficiency of a particular lysosomal enzyme but rather to a defect in endocytosis (Bargal and Bach 1997; Chen et al. 1998). It has been suggested that many cases may not be properly diagnosed, because of the variability in the observed phenotype and the lack of an indicative storage compound.

The MLIV locus was mapped to chromosome 19p13 in AJ families, and haplotype analysis of these families indicated that there were two founder chromosomes that accounted for 73% (major haplotype) and 23% (minor haplotype)—a total of 96%—of the carrier chromosomes in the AJ population (Slaugenhaupt et al. 1999; Sun et al. 2000). Recently, the gene that is mutated in patients with MLIV, *MCOLN1* (MIM 605248), was isolated, and the two AJ founder mutations were identified. These mutations were (1) a splicing mutation, IVS3−2A→G, which resulted in skipping of exon 4, with the subsequent loss of the reading frame, and (2) a 6,434-bp deletion spanning genomic nucleotides 511–6,944, designated "511del6434," which included the 5′ flanking region of the gene through exon 7 (Bargal et al. 2000; Bassi et al. 2000; Sun et al. 2000). Since both mutations resulted in the loss of a functional gene product, the severity of the phenotype was similar in patients who were homoallelic or heteroallelic for these mutations.

The *MCOLN1* gene encodes the putative protein mucolipin-1, which is 580 amino acids long and is somewhat homologous to the polycystins, a family of cation channels (Somlo and Ehrlich 2001). In *Caenorhibditis*

Received December 10, 2001; accepted for publication January 7, 2002; electronically published February 13, 2002.

Address for correspondence and reprints: Dr. Ruth Kornreich, Department of Human Genetics, Box 1497, Mount Sinai School of Medicine, New York University, Fifth Avenue at 100th Street, New York, NY 10029. E-mail: ruth.kornreich@mssm.edu

0002-9297/2002/7004-0021$15.00

elegans, studies of cup-5, the mucolipin-1 homolog, have indicated that these proteins are vesicle membrane–associated regulators of the endocytic pathway, where loss of cup-5 function results in large lysosomal or late endosomal structures that exhibit both an increase in the rate of uptake of fluid phase markers and a decrease in the ability to degrade proteins (Fares and Greenwald 2001). The function of mucolipin-1 that is predicted on the basis of these studies is consistent with the lysosomal pathology observed in cultured cells derived from patients with MLIV.

To determine the frequency of carriers for MLIV in the AJ population, we screened for both the major IVS3−2A→G and the minor 511del6434 mutations in genomic DNAs isolated from blood samples obtained from 2,029 unrelated AJ individuals from the New York metropolitan area who were referred for prenatal carrier testing for other genetic disorders prevalent in the AJ population. DNA was extracted from peripheral blood obtained by venipuncture and archived with informed consent for use in research studies. All personal identifiers were removed, and the samples were tested anonymously. A multiplex PCR amplification was performed for 28 cycles with 100 ng of genomic DNA in a volume of 50 μl containing 10 pmol of each primer tagged with a 5′ universal primer sequence (UPS) (Shuber et al. 1995). The primer sequences designed to detect the major mutation were MLIV-1UPS (5′-GCGGTCCCAAAAGGGTCAGTATCTTCCATGCTGTGGACCA-3′) and MLIV-2UPS (5′-GCGGTCCCAAAAGGGTCAGTAACAGTGAAGCCTCGTCCTG-3′), and the primer sequences designed to detect the minor mutation were MLIV-3UPS (5′-GCGGTCCCAAAAGGGTCAGTGGCAGCTTTCTCAATGAAGG-3′ and MLIV-4UPS (5′-GCGGTCCCAAAAGGGTCAGTTCACCGTGCTGGAAGACACT-3′). In addition, 100 mM of each dNTP (Roche Molecular Biochemicals), 5 U of *Taq* DNA polymerase (Roche), 10 mM Tris-HCl, 50 mM KCl, 0.1% TritonX-100, and 1.5 mM $MgCl_2$ were added to the PCRs. Dimethyl sulfoxide (Sigma) was added to a final concentration of 4%, because of the high (65%) GC content of the region amplified for analysis of the major mutation. The primers were designed from the genomic sequence of the *MCOLN1* gene (Sun et al. 2000; GenBank accession number AF287270) to amplify a 410-bp PCR product encompassing the IVS3−2A→G mutation and a 396-bp PCR product when a 511del6434 mutation was present (fig. 1). Therefore, the multiplex PCR amplification of DNA from individuals with neither mutation and individuals who were heterozygous or homozygous for the IVS3−2A→G mutation would result in a single PCR product of 410 bp (fig. 1). Amplification of DNA from individuals who were carriers of the 511del6434 mutation or heteroallelic for both mutations would result in PCR products of 396 bp and 410 bp, respectively, whereas amplification of DNA from individuals who were homozygous for the 511del6434 mutation would result only in products of 396 bp (fig. 1). Control DNAs were obtained from the fibroblast cell lines GM02527, GM02529, and GM02525 (Coriell NIGMS Human Genetic Cell Repository), which were derived from patients with MLIV who were homozygous for the IVS3−2A→G mutation, compound heterozygous for the IVS3−2A→G/511del6434 mutations, and homozygous for the 511del6434 mutation, respectively (Bassi et al. 2000).

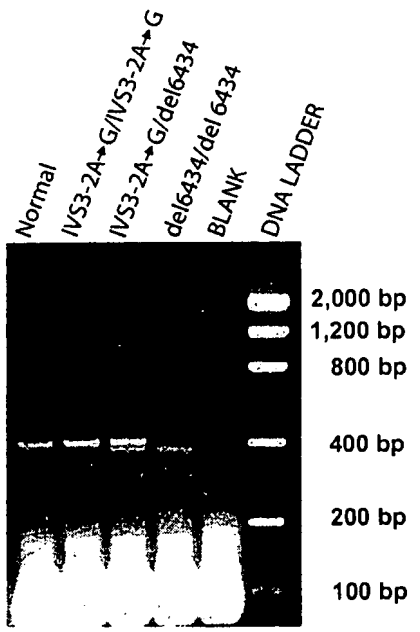

Figure 1 Multiplex PCR of the *MCOLN1* gene; 2% agarose gel showing the products that result from the multiplex PCR amplification of the *MCOLN1* gene with the IVS3−2A→G and 511del6434 primer pairs. Note that the 511del6434 primers amplify a product only when the deletion is present.

Aliquots (4 μl) of the PCR products were blotted in triplicate onto 8 × 12 cm Hybond −N+ membranes (Amersham Pharmacia Biotech) through use of a Biomek 2000 automated pipetting workstation (Beckman Coulter). Hybridization was performed with the following allele-specific oligonucleotides (ASOs): for the IVS3−2A→G mutation, 5′-TCTCTGCCCACAGTACCTG-3′ (normal) and 5′-TCTCTGCCCACGGTACCTG-3′ (mutant); and for the 511del6434 mutation, 5′-CCTGGGCTCAACAAAGCAC-3′. For radioactive detection, the membranes were hybridized, for a length of time ranging from 2 h to overnight, with ~10^6 counts per minute of γ-[^{32}P]ATP end-labeled probe/ml and 10-fold molar excess of the mutant or wild-type competitor oligonucleotide for the IVS3−2A→G mutation. The membranes were then washed sequentially in 5 × saline sodium citrate (SSC) for

5 min at room temperature and in 5 × SSC for 5 min at 45°C, followed by a wash in 0.1 × SSC/0.1% SDS for 5 min at 45°C. Filters were then exposed to autoradiography by use of Biomax MR film (Kodak) (fig. 2).

For the major IVS3−2A→G mutation, 2,029 AJ individuals were screened and 11 carriers were identified, corresponding to a frequency of 0.54%, or 1/184. Five carriers for the minor 511del6434 mutation were identified in a total of 2,029 AJ individuals tested, corresponding to a frequency of 0.25%, or 1/406. The combined frequency of the two mutations in the present study was, therefore, 0.79%, or 1/127 individuals (95% CI 0.40%–1.17%) (table 1). Because the haplotypes that correspond to these two mutations account for 96% of the carriers in the AJ population, the prevalences of the two mutations were 66% and 30% for the major and minor mutations, respectively. These percentages are in agreement with the results of Sun and colleagues (2000), who reported that the major and minor haplotypes are present on 73% and 23% of carrier chromosomes, respectively.

In a recent report (Wang et al. 2001), a small study of 123 AJ individuals was conducted, and a carrier frequency of 1/61 was reported for the major IVS3−2A→G mutation; however, no carriers for the minor 511del6434 mutation were identified (table 1). Therefore, the limited sample size prevented a reliable estimate of the frequency and distribution of MLIV carriers. Recently, Bargal et al. (2001) reported a carrier frequency of 1/100 for the two mutations in a sample of 2,000 AJ individuals from Israel (table 1). The distribution of the mutations was 94% for the major mutation and 6% for the minor mutation. This apparent disparity in the frequency of carriers between the AJ constituencies of the New York metropolitan area and Israel is not clear; however, the major mutation is more prevalent in the Israeli population and probably accounts for the observed higher frequency of total carriers of MLIV. These findings also may be indicative of different patterns of immigration of AJ individuals into the United States and Israel and/or may reflect distinct regional origins of the founders of the major and the minor mutation.

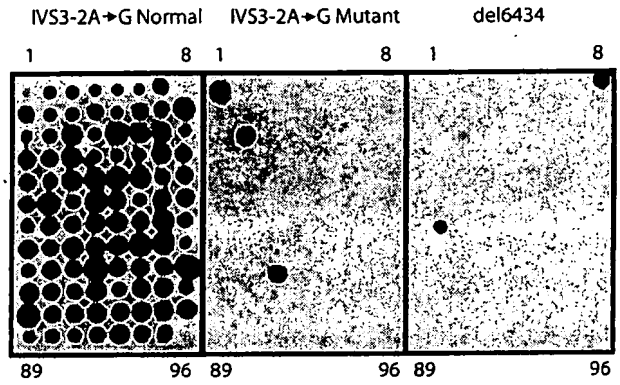

Figure 2 ASOs of the two AJ *MCOLN1* mutations. Shown are autoradiographs of dot blots hybridized with the normal IVS3−2A→G ASO (*left*), the mutant IVS3−2A→G ASO (*middle*), and the 511del6434 ASO (*right*). PCR samples 1–95 were amplified from genomic DNAs; sample 96 is the ddH_2O control. Sample 1 was amplified from the IVS3−2A→G homozygote (GM02527), sample 8 was amplified from the 511del6434 homozygote (GM02525), samples 18 and 67 were amplified from carriers of the IVS3−2A→G mutation, and sample 49 was amplified from a carrier of the 511del6434 mutation.

In a study of 17 Israeli AJ families with MLIV, familial origins were traced back to Poland or to neighboring Lithuania (Raas-Rothschild et al. 1999). The authors speculated that the underrepresentation of ultra-orthodox families among the 80 AJ families with MLIV worldwide might indicate a recent origin for the mutation, around the 18th and 19th centuries, in a founder that belonged to a secular family. These findings are likely to be specific for the major mutation, on the basis of its higher prevalence (94%) among the Israeli AJ population. Discordant carrier rates between Israeli and American Ashkenazi subpopulations have been reported recently for mutations in the *Connexin 26* gene, which cause nonsyndromic sensorineural recessive deafness

Table 1

Carrier Frequency of the *MCOLN1* Mutations Among Unrelated AJ Individuals

Study (Population)	No. of Individuals	IVS3−2A→G		511del6434		Combined	
		No. of Carriers	Carrier Percentage (Frequency)	No. of Carriers	Carrier Percentage (Frequency)	No. of Carriers	Carrier Percentage (Frequency)
Bargal et al. 2001 (Israel)	2,000	17	.85 (1/117)	1	.05 (1/2,000)	19[a]	.95 (1/106)[b]
Wang et al. 2001 (New York)	123	2	1.62 (1/62)	0	0	2	1.62 (1/62)
Present study (New York)	2,029	11	.54 (1/184)	5	.25 (1/406)	16	.79 (1/127)[c]
Combined	4,152	30	.72 (1/138)	6	.14 (1/692)	37	.89 (1/112)

[a] This number is based on an estimate of a 95% detection rate of MLIV carriers.
[b] 95% CI = .52%–1.39%.
[c] 95% CI = .40%–1.17%.

(Dong et al. 2001). Variation in the frequency of MLIV carriers between the United States and Israel thus provides further evidence that Ashkenazi subpopulations have not reached equilibrium.

A more recent origin for the two AJ MLIV founder mutations would explain why the frequency of carriers for MLIV is not as high as the frequencies of other mutations that are common in the AJ population, including some that originated in or were introduced into the European Jewish population >1,000 years ago (Diaz et al. 2000). The carrier frequencies of these disorders range from 1/18, for Gaucher disease (Beutler and Grabowski 2000), to 1/107, for Bloom syndrome (Li et al. 1998). The observed frequency of MLIV carriers within a large cohort of AJ individuals from the New York metropolitan area is 1/127, slightly lower than the frequency of disorders for which carrier screening is currently available. However, in light of the neurologic severity of the MLIV phenotype, for which there is no available treatment, and the fact that the two mutations account for >95% of carriers, carrier screening for the two mutations should be considered for this population.

Acknowledgments

The authors would like thank all the individuals who agreed to participate anonymously in this study. We also thank Asghar Bajwa and Jing Xu for their technical assistance. This work was supported, in part, by grants from the National Institutes of Health, including a Merit Award (grant 5 R37 DK34045), grant 5 M01 RR00071 to the Mount Sinai General Clinical Research Center, and grant 5 P30 HD28822 to the Mount Sinai Child Health Research Center.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genbank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *Homo sapiens MCOLN1*, complete coding sequence [accession number AF287270])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for MLIV [MIM 252650] and mucolipin-1 [MIM 605248])

References

Amir N, Zlotogora J, Bach G (1987) Mucolipidosis type IV: clinical spectrum and natural history. Pediatrics 79:953–959

Bargal R, Avidan N, Ben-Asher E, Olender Z, Zeigler M, Frumkin A, Raas-Rothschild A, Glusman G, Lancet D, Bach G (2000) Identification of the gene causing mucolipidosis type IV. Nat Genet 26:118–123

Bargal R, Avidan N, Olender T, Ben Asher E, Zeigler M, Raas-Rothschild A, Frumkin A, Ben-Yoseph O, Friedlender Y, Lancet D, Bach G (2001) Mucolipidosis type IV: novel MCOLN1 mutations in Jewish and non-Jewish patients and the frequency of the disease in the Ashkenazi Jewish population. Hum Mutat 17:397–402

Bargal R, Bach G (1997) Mucolipidosis type IV: abnormal transport of lipids to lysosomes. J Inherit Metab Dis 20: 625–632

Bassi MT, Manzoni M, Monti E, Pizzo MT, Ballabio A, Borsani G (2000) Cloning of the gene encoding a novel integral membrane protein, mucolipin-1, and identification of the two founder mutations causing mucolipidosis type IV. Am J Hum Genet 67:1110–1120

Berman ER, Livni N, Shapira E, Merin S, Levij IS (1974) Congenital corneal clouding with abnormal systemic storage bodies: a new variant of mucolipidosis. J Pediatr 84:519–526

Beutler E, Grabowski GA (2000) Gaucher's disease. In: Beaudet A, Childs B, Kinzler K, Scriver CR, Sly WS, Valle D, Vogelstein B (eds) The metabolic and molecular bases of inherited disease, 8th ed. McGraw-Hill, New York, pp 3635–3668

Chen CS, Bach G, Pagano R E (1998) Abnormal transport along the lysosomal pathway in mucolipidosis type IV disease. Proc Nat Acad Sci USA 95:6373–6378

Diaz GA, Gelb BD, Risch N, Nygaard TG, Frisch A, Cohen IJ, Miranda CS, Amaral O, Maire I, Poenaru L, Caillaud C, Weizberg M, Mistry P, Desnick RJ (2000) Gaucher disease: the origins of the Ashkenazi Jewish N370S and 84GG acid β-glucosidase mutations. Am J Hum Genet 66:1821–1832

Dong J, Katz DR, Eng CM, Kornreich R, Desnick RJ (2001) Nonradioactive detection of the common *Connexin 26* 167delT and 35delG mutations and frequencies among Ashkenazi Jews. Mol Genet Metab 73:160–163

Fares H, Greenwald I (2001)Regulation of endocytosis by CUP-5, the *Caenorhabditis elegans* mucolipin-1 homolog. Nat Genet 28:64–68

Li L, Eng C, Desnick RJ, German J, Ellis NA (1998) Carrier frequency of the Bloom syndrome *blm*^{Ash} mutation in the Ashkenazi Jewish population. Mol Genet Metab 64:286–290

Raas-Rothschild A, Bargal R, DellaPergola S, Zeigler M, Bach G (1999) Mucolipidosis type IV: the origin of the disease in the Ashkenazi Jewish population. Eur J Hum Genet 7: 496–498

Riedel KG, Zwaan J, Kenyon KR, Kolodny EH, Hanninen L, Albert DM (1985) Ocular abnormalities in mucolipidosis IV. Am J Ophthalmol 99:125–136

Shuber AP, Grondin VJ, Klinger KW (1995) A simplified procedure for developing multiplex PCRs. Genome Res 5:488–493

Slaugenhaupt SA, Acierno JS Jr, Helbling LA, Bove C, Goldin E, Bach G, Schiffmann R, Gusella JF (1999) Mapping of the mucolipidosis type IV gene to chromosome 19p and definition of founder haplotypes. Am J Hum Genet 65: 773–778

Somlo S, Ehrlich B (2001) Human disease: calcium signaling in polycystic kidney disease. Curr Biol 11:R356–R360

Sun M, Goldin E, Stahl S, Falardeau JL, Kennedy JC, Acierno JS Jr, Bove C, Kaneski CR, Nagle J, Bromley MC, Colman M, Schiffmann R, Slaugenhaupt SA (2000) Mucolipidosis type IV is caused by mutations in a gene encoding a novel transient receptor potential channel. Hum Mol Genet 9: 2471–2478

Wang ZH, Zeng B, Pastores GM, Raksadawan N, Ong E, Kolodny EH (2001) Rapid detection of the two common mutations in Ashkenazi Jewish patients with mucolipidosis type IV. Genet Test 5:87–92

1: AF287270. Reports Homo sapiens muco...[gi:9844925] Links

- Features
- Sequence

```
LOCUS       AF287270               13270 bp    DNA     linear   PRI 30-OCT-2000
DEFINITION  Homo sapiens mucolipin (MCOLN1) gene, complete cds.
ACCESSION   AF287270
VERSION     AF287270.1  GI:9844925
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Catarrhini;
            Hominidae; Homo.
REFERENCE   1  (bases 1 to 13270)
  AUTHORS   Sun,M., Goldin,E., Stahl,S., Falardeau,J.L., Kennedy,J.C.,
            Acierno,J.S. Jr., Bove,C., Kaneski,C.R., Nagle,J., Bromley,M.C.,
            Colman,M., Schiffmann,R. and Slaugenhaupt,S.A.
  TITLE     Mucolipidosis type IV is caused by mutations in a gene encoding a
            novel transient receptor potential channel
  JOURNAL   Hum. Mol. Genet. 9 (17), 2471-2478 (2000)
   PUBMED   11030752
REFERENCE   2  (bases 1 to 13270)
  AUTHORS   Slaugenhaupt,S.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-JUL-2000) Molecular Neurogenetics, Harvard Institute
            of Human Genetics, 77 Ave. Louis Pasteur, HIM Building Room 422,
            Boston, MA 02115, USA
FEATURES             Location/Qualifiers
     source          1..13270
                     /organism="Homo sapiens"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:9606"
                     /chromosome="19"
                     /map="19p13.3"
     gene            1401..12753
                     /gene="MCOLN1"
     mRNA            join(1401..1556,3736..3941,5214..5381,5536..5701,
                     6295..6403,6639..6735,6933..7032,7372..7478,7596..7745,
                     7876..7977,8365..8487,9061..9276,12298..12428,
                     12534..12753)
                     /gene="MCOLN1"
                     /product="mucolipin"
     CDS             join(1526..1556,3736..3941,5214..5381,5536..5701,
                     6295..6403,6639..6735,6933..7032,7372..7478,7596..7745,
                     7876..7977,8365..8487,9061..9276,12298..12428,
                     12534..12570)
                     /gene="MCOLN1"
                     /codon_start=1
                     /product="mucolipin"
                     /protein_id="AAG00798.1"
```

/db_ f="GI:9844926"
/translation="MTAPAGPRGSETERLLTPNPGYGTQAGPSPAPPTPPEEEDLRRR
LKYFFMSPCDKFRAKGRKPCKLMLQVVKILVVTVQLILFGLSNQLAVTFREENTIAFR
HLFLLGYSDGADDTFAAYTREQLYQAIFHAVDQYLALPDVSLGRYAYVRGGGDPWTNG
SGLALCQRYYHRGHVDPANDTFDIDPMVVTDCIQVDPPERPPPPPSDDLTLLESSSSY
KNLTLKFHKLVNVTIHFRLKTINLQSLINNEIPDCYTFSVLITFDNKAHSGRIPISLE
TQAHIQECKHPSVFQHGDNSFRLLFDVVVILTCSLSFLLCARSLLRGFLLQNEFVGFM
WRQRGRVISLWERLEFVNGWYILLVTSDVLTISGTIMKIGIEAKNLASYDVCSILLGT
STLLVWVGVIRYLTFFHNYNILIATLRVALPSVMRFCCCVAVIYLGYCFCGWIVLGPY
HVKFRSLSMVSECLFSLINGDDMFVTFAAMQAQQGRSSLVWLFSQLYLYSFISLFIYM
VLSLFIALITGAYDTIKHPGGAGAEESELQAYIAQCQDSPTSGKFRRGSGSACSLLCC
CGRDPSEEHSLLVN"

ORIGIN

```
       1 tctcacttac cccttgctct tcaaagccca tacagtaggt atacaagtgg acaaaaaaag
      61 ttgctcattt atgcaatcaa caaacatctc tggattgctg gggtctcagc agggaacaag
     121 ataaatatgg cctcgacctg catggagctc atagatacta aattcagaat acttaaaaaa
     181 taattacggg gtatagtaca ttctaggaga agcataacaa gacttctgat ataaatggca
     241 ggcagctttc tcaatgaagg attttgtaat cccaataatc actaatttaa taatcagtac
     301 tgtttgccca gccttatgcg atagtttttg cattctctca tttaatcctc tcaacagccc
     361 cagtaggtag atgactttga atatccccat tttgcaaatg agaaaattga ggcacatttt
     421 ttttttttt tttagacagt cttgctctgt tgcccaggct ggagtgcagt ggtgtgatca
     481 tagctcactg cagcctcgac ctcctgggct caagcgatcc tcccacctta gcctcccgag
     541 tagctgggat tgccggtgca tgccaccgcc cactgcgctc agcttggagt tgaagggact
     601 ctggaagatg tagaagtggc attgtcagtg cctagattta aatcccaatt gccctccagg
     661 gtccaaattc ttaaccatta cgctccaggg caaaagtatg caaaggctct ggggctatag
     721 aaagatgagc tttggatgga ggtaggagcc agatcagagg gccctgatag acgagagtgg
     781 ggactctgcc tgtcattaca gagcaatggg aagccgaggg caggttctcg caggaaggat
     841 aggaattatt ctttgaagat gcttgtggct gctgggtaga gagtggagtg gagggaggct
     901 gagatcgggg aggaggttgc tgcaaagatc caggccagga atgttggaag actctgggct
     961 gggggccatg gggtggggat aagtggttct atttgataca taattaggaa atcgtgtttg
    1021 ctgaagatgc gcaggagaag ggtaaaagga gtttctggga gaaagaggaa gacagcgttg
    1081 agatagtagg cagggtcatc accaggcacc aaggaggata aggggtcaag ctctggacat
    1141 ggaagtcaca agcctggcac cggattcggg gcatggccgg gagccagggc agagctcgtc
    1201 gttgccaaac tcagagtcag cccatccccc gccacccaga gcgcgtcggc gctaggacct
    1261 agcgactgcc ttcgacccag agggcgccgg cagaggcacg catgcgcgct gttccggcag
    1321 gggttgtcgt ggcgcagggg gcgggaccag aggcggtcac gtgaggggct ctgggctacc
    1381 gggtcacgtg accgaggcac agatcagctg atgccggagg gtttgaagcc gcgccgcgag
    1441 ggagcgaggt cgcagtgaca gcggcgggcg atcggaccca ggctgccccg ccgtacccgc
    1501 ctgcgtcccg cgctcccgcc ccagcatgac agccccggcg ggtccgcgcg gctcaggtga
    1561 gggcgcgggc ggcaccgtgg ggccccgaac tcaggcgggc gggctgtgtc tcccacctgg
    1621 ggcggcggag ctcctagtct ctttttttct aagctccagc gctgactttt cacggtggag
    1681 aaaagggcag acggctccta gaacttgggc ggcgggtggg caccagcctc tccaattctt
    1741 cctcctgaac ccaggctctg ctgggttccc aaactcaggc agggatcgcg ccgggccgcc
    1801 agcttctccc tctggggcgg cgaggttcct gggattccca ctgggagcct aggttccgat
    1861 tgctcaactt cgtctggaac tcagacagcg ggcaccagct tctccaaccc gcacgtgaga
    1921 ctcccaggct tcccctcctg attccagggg acaaatgctc agcttcccta agctcaagcc
    1981 tggagagctg gagggattgc ccccaggcga ttaactcagt tttagctttc caaaccgctg
    2041 gaagcgcagc cttcttaaat tcgggcttct agccaattct gatgccaccc ctcctcgggg
    2101 aggctggagg aagacccctt gtgttagctt ccccttctgg agctagctgg ggacccctac
    2161 ctgatagatg tcccggtgtc ccagctagta gggtctgggg tgggttagct gtaatctcag
    2221 ctctgtaagc gggccctgcc ctctggcttt gtcgtaaaca gccacagcag catctcattg
    2281 caaagggagg ggccgggaac ttgtccctct ctgcaaggga ggttctgaca gtgcacacat
    2341 ttatcctgac tgctttgcta ggcaggaggc caggccctag aaagcagcac ggggccaggc
    2401 cctagaaagc acatccccat gggggtgtga cagggacagt tttgggctac tgtgactggt
    2461 tttgactcca gcagttgctg aaagcttaga tctaaccatt aggctggaaa aaaataaaca
    2521 gtgattagaa cagcttgtgt ttgctgaaga ggtctttatc tgctgtgtct cactgaattc
    2581 tcagagcagc ttcaggatct caacctcaag gctcagggag agggtggact ttttttttt
    2641 ttaataaact ttttttgtt gcccaggctg tagtgcagtg gcataatcct agctcattgt
    2701 aacatcgaac tcctgggctc aagtgatcct cccaactcag cctcccgggt agatggggtc
    2761 ccagctacta actacgggca tgagccgtca cacctgacta tttaaaaaaa atgttttttt
    2821 tttgtagaca gggaggtctc gctgtattac ctaggctgga tcctcccacc ttggcctccc
    2881 aaagccgttg ggataacagg catgagccac tgagcccagc caaggggtcg ccttttaaa
    2941 atttccactc ttcagatgag gagatggagg ctcagggagg tacctggagt caacctactg
    3001 taaagtggca ggtctgggat ttgatgctag ggctgcatga tttctaggag ctggtgcttt
```

```
3061 tcagggagat aaaa  gtc tttagcgaat gtgttccatt atta  ctt atgttgtcaa
3121 ttacctcttc tccaggtcct tggcttctga gagtgtcagc tgatgggcca ggttataatg
3181 aacccagagg tcatcttttg ggtatttgtc cagacaaacc tagaatacag gctgagttct
3241 atgctcatgt ctggaagctg gagttgggat aagcccagca ggcttgaacg cccagtgaaa
3301 agccagtggg agcagttcat tctctcccca ctgatcaata acgggaacat tgatgaaatg
3361 ttctgacatt caccatggac cagcccctgt gatcaatgct tcataagcat ccagtcctta
3421 gcgttcccat gagacatatt attgccccat ttcgcagatg aggaaactga ggctcagaga
3481 gctggtgagc aggaggggca ggaatcagcc caggccctgt acctcccaaa cccaaactca
3541 taacctctga gcaggacggg tgcatagata cctacaatgt cacaggtttt ctggttttct
3601 ttagacctct cagagctctt ccttggcagg agcatgggga catgaagata gggcgtgtgc
3661 tgccttcctg gttggagaaa ggggaaaagg ggagttgccc aggcctcacc ccagtgccct
3721 ctcctattcc cacagagacc gagcggcttc tgacccccaa ccccgggtat gggacccagg
3781 cggggccttc accggccccc ccgacacccc cagaagagga agaccttcgc cgtcgtctca
3841 aatacttttt catgagtccc tgcgacaagt ttcgagccaa gggccgcaag ccctgcaagc
3901 tgatgctgca agtggtcaag atcctggtgg tcacggtgca ggtgaggcca gccaagcagg
3961 ggccccagct gaaggccacc tgtggctgct gtgctccttg aagagagtct taaagcagca
4021 ctttggaagg ccgaggccgg tggatcgctt gaggctggga gttcaagacc agtctggcca
4081 gcatggtgaa accccatctc tactaaaaat acaaaaaaat tagccgtgcg tggtggcggg
4141 tgcctgtaat cccagctact tggcaggctg aggcaggaga atcgcttgaa ttgggaggcg
4201 gaggttgccg tgagctgaaa tcatgccact gcactccagc ctgggcaaca gagcaagact
4261 gtctcaaaaa aaaaaagaag ccgactctga ggctcagaga ggttaggaga cttgcccaaa
4321 gtcacacagc aatagaacat tgggagctgg gatttgaacc caggcagtct gacaccatgt
4381 tgacccaatg gctgcacaga tagttctccc tcccccatgc cagaccctgt gctgggctct
4441 gggaacccca agatgaatca gacccagcca ctgccctaag tgcttacttc atgttttggg
4501 ctgactttag catgtcacca tgcctctaat tttccctctg aaaagggacc caattgtcca
4561 ggcatggtgg ctcatgcctg taatgccagc actttgggag gctgagttgg gtggatcatt
4621 tgaggccagg agtttgagac cagcctggcc aacattgcaa aaccccgtct ctactaaaaa
4681 tacaaaaatt agctgggttt ggtggcaggt acctgtaact cagctactca ggaggctgag
4741 acaggagaat tgcttgaacc caggggggtgg aggttgtagt gagctgagat cataccatgg
4801 cactccaact tgggcaacag agtgagactc tgtctcaaaa aagaaaagaa aagggaccca
4861 gtcatggtac ttaccctgaa agtttgggtt taacacagaa tcggacatcc agtaaacatt
4921 taatgaacgt tagtccctgc agtgagatag atgagtcccc accctgtgtt gtacggggga
4981 ggacacagtg gtgggcgtgg catggagctt atgccaggag gtggggtgaa attaatcaaa
5041 gcaaagaaat gcacaagtga aatccgtgtt tgtggcccaa gttagcaggg ccctgcccca
5101 ccccagtgga catctgcagg gccctccctg tcctcttcca gggcctgtgc cctgagggag
5161 atacacccca acccccatcc tagccatgcc aacctctact accctctccc cagctcatcc
5221 tgtttgggct cagtaatcag ctggctgtga cattccggga agagaacacc atcgccttcc
5281 gacacctctt cctgctgggc tactcggacg gagcggatga caccttcgca gcctacacgc
5341 gggagcagct gtaccaggcc atcttccatg ctgtggacca ggtgctggtg ggcgggcagg
5401 tgctggtggg caggcaggtg caggtgggcg ggcaggtgca gttgggcggg caggtgctgg
5461 tgggcgggca ggtgcaggtg ggtgggctgc agagagcggg ccggactcac aggccctccc
5521 cttctctgcc cacagtacct ggcgttgcct gacgtgtcac tgggccggta tgcgtatgtc
5581 cgtggtgggg gtgacccttg gaccaatggc tcagggcttg ctctctgcca gcggtactac
5641 caccgaggcc acgtggaccc ggccaacgac acatttgaca ttgatccgat ggtggttact
5701 ggtgagtggg caggacgagg cttcactgtt gggagcctga gctgctggga ttaaaatcaa
5761 cagctgtggc tgggcacggt ggctcacgcc tataatacca gcactttggg aggctgagga
5821 ggaaggattg cttgaggcca gaagtttgag accagcctgg gccacgtagg aagaccttgt
5881 ctctacgcac aaacaaatta gctgggcgtg gtggcgtgcc cctgtggtcc cagctactca
5941 ggaggctgag gcaggaggat cgcttgagtc cgggaggttg aggctgcagt aagctatgac
6001 cacgctgctg cactccaccc tgggtgacag agtgagaccc tgtctcaaaa aaaaaaaaaa
6061 aaaaaaaaaa caagtatgct tagtgtgagt gtgactcttg ccacgtagaa agcaccagat
6121 gttatatttt aatatggctc attcagtaaa acatccgcag gcccagagag tgccaggcct
6181 gtaggaatga cccaaccctg gggaagcaca gggaagaagg ccactgggga ctctggggag
6241 accagcctgg cctccccggc cccctgaggc ccttccctga ctccctgtcc ttagactgca
6301 tccaggtgga tccccccgag cggcccctc cgcccccag cgacgatctc accctcttgg
6361 aaagcagctc cagttacaag aacctcacgc tcaaattcca caagtactgc ctgctcactc
6421 gaggggggcc cagggtgggg gaggcagcac actaggcact ctcaccccag caactacttc
6481 cctaaggtgg ggacagggcc cccccgcccg cgctggtgcc tgctgggtga gcacttcccc
6541 tgccagctgc agagtcagca cgtggcaggg gacgctggca cttggggccg gaagggaccc
6601 gaagacgccc ctgaccctca cccgagcctc ctgcctaggc tggtcaatgt caccatccac
6661 ttccggctga agaccattaa cctccagagc ctcatcaata atgagatccc ggactgctat
6721 accttcagcg tcctggtgag gccccccggg aacccacagg gctcctgagt tccagggcag
6781 ggacctggtc agggagtgtc ttgggagcac tggccaaggg caagcgtgcg ggtgatgagg
6841 gagggagccc ggggtctgtc aggccacctg tcatgtggac cttggggctt ggggctgcca
```

```
 6901 aggtttactc tgcc   aac tggcccccac agatcacgtt tgac   aaa gcacacagtg
 6961 ggcggatccc catcagcctg gagacccagg cccacatcca ggagtgtaag caccccagtg
 7021 tcttccagca cggtgagccc ctgagcccca gaccagcact gaccaggggc cctggcctgt
 7081 cctgggattc cccaagcccc agatcagcgc tgcctggggg ccgtgacctc cccaggaatc
 7141 cgctgagcct cagatcagca cagaccaggg accccgtcct gtgctgagat cccccaagcc
 7201 ccagaccagc actgaccggg gttcttgact caccccaagc aagccctgag cccactgacc
 7261 aaccaaaacc agccgtgcag ccccctaggt ctccagcctg gcctggcacc aatgctagcc
 7321 tcccaaggct ccatgccatc cttggcccta cccgctctgc cctccccgca ggagacaaca
 7381 gcttccggct cctgtttgac gtggtggtca tcctcacctg ctccctgtcc ttcctcctct
 7441 gcgcccgctc actccttcga ggcttcctgc tgcagaacgt gaggcttctg cgtcatgtgt
 7501 gctggtgtcc tccccgcctg gccctggggc gataaaagcc agggctttga gggtcctgtg
 7561 cctggtcagg ccctcacccc gcctgccttc tgcaggagtt tgtggggttc atgtggcggc
 7621 agcggggacg ggtcatcagc ctgtgggagc ggctggaatt tgtcaatggc tggtacatcc
 7681 tgctcgtcac cagcgatgtg ctcaccatct cgggcaccat catgaagatc ggcatcgagg
 7741 ccaaggtgcg tcctgccaac accctgggcc ccaggtccca tccctgctgt cagtgcctat
 7801 ccggggccat atcctccccc aggcccccca aaggaagggc tgggccagat aggttgacgc
 7861 agctcccacc cgcagaactt ggcgagctac gacgtctgca gcatcctcct gggcacctcg
 7921 acgctgctgg tgtgggtggg cgtgatccgc tacctgacct tcttccacaa ctacaatgtg
 7981 agttttgcac atgcagctgg gccttccaca tggttactcc acaccctcca aataaatccc
 8041 tacacacgca gccctcacca gccccggcca atggcccctt gcaagcctcc tcctcctacc
 8101 tgcccacacc agatatatct gtcactgcac ctgcgcgggg ccccgggagc ctgctccttt
 8161 gtgcccaccc agctgagtct agccgtgcgt tgccctcgga ccccctcaga cgtggccacg
 8221 ccccctctag gcacccactg gctcccatga ccacaccggc tgtgccctcg gcaaggcccc
 8281 gcccctccca accccatctg ggtgcccaca gctgacctga gttgtggcca caccctcaac
 8341 gaggctccct ctgccccaac ccagatcctc atcgccacac tgcgggtggc cctgcccagc
 8401 gtcatgcgct tctgctgctg cgtggctgtc atctacctgg gctactgctt ctgtggctgg
 8461 atcgtgctgg ggccctatca tgtgaaggta catctaaccc ctgatgtccc tgacattgac
 8521 cctgtgacct tgtcattgac actgtgaccc ccagatgacc ccttggtgac tgctgggagt
 8581 ctgtccactg tcccctgtgg tccttggtga ccctgacact gaccctgtgc cattattgtt
 8641 gtcacagttg ttgatgaccc tatttcgacc tgaattactc ccctcctgct ctatctaccc
 8701 agaccctagg tcggccctgt ggccctgtca ttgacccgtg gtcccggcca ttcacatggg
 8761 accccagcct gggacctggc cattcacata gtgaccccag cctgggaccc ggccattcac
 8821 gtgggacccc agcctgggtc ccggccattc acgtgggacc ccagcctggg acccggccat
 8881 tcacaggggc cctagcctgg aacccgacca ttcacatggt gaccgcagcc cgggacccgg
 8941 ccattcatgt ggggccccag ccaccagctc ctagccattt gcatgggacc ccagcctgac
 9001 cccagccccc ggttcctggc catgccttgg ctccctctga ccccgccgcc cctctggcag
 9061 ttccgctcac tctccatggt gtctgagtgc ctgttctcgc tcatcaatgg ggacgacatg
 9121 tttgtgacgt tcgccgccat gcaggcgcag cagggccgca gcagcctggt gtggctcttc
 9181 tcccagctct acctttactc cttcatcagc ctcttcatct acatggtgct cagcctcttc
 9241 atcgcgctca tcaccggcgc ctacgacacc atcaaggtca gccgcatgca cccagccctg
 9301 agctcgggct ctgggtgccc tggagtctgc catgaggggg tcttggggac accgcagggt
 9361 gaacagagaa gacccaggag agaatatggg agactctatg aaaccaaaaa gagggtggtt
 9421 cagaactggg gggcgcaggg ggatgtcaag gtgggcttgg gccaggaggg ggcctgagtc
 9481 agtctttgcc aacagggcaa ccgagtcata gagtttattt atttatttgt ttatttgaga
 9541 cggagtcttg ctctgtcacc cagggtggag tgcagtggtg cgatcttgac tcactgcaac
 9601 ctccacctcc cgggttcaag caattctgtc tcagcctcct gagtagctgg gactacaggc
 9661 acacgccacc acgtccagct aatttttgta tttttagtag agatggcatt tcaccgcatt
 9721 ggtcaggctg gtctcaaact cctggcctca ggagatctac tgccttggcc tcccaaagtg
 9781 ttgggattac aggcgtgagc caccacgccc ggcctatttt attttattat taaagtattg
 9841 ttctttattt tattagagac aagggtctca ctgtgttacc caggctggtt tcaaactcct
 9901 gaggtcaagt gatcctccca ctttggcctc ccaaagtgct gggattacag gcgtaagcca
 9961 ccacacccag cctattatta ttatttttt tttgaaatgg aatcttaccc tgtggcccag
10021 gctggagtgc aatggcatga tctcggctca ctgcaacctc caccttctga gttgaagcga
10081 tccttgtgcc tcagcctcct gagtagctgg gattacttgc acgtgccacc acacctggct
10141 aatttttgta tttttactag agatggggtt tcaccacgtt ggccaggctg gtctcgaact
10201 cctgacctca ggtgatccac ctgccttggc ctcccaaggt gctgggattt caggcatgag
10261 ccactgaacc cagctaagtc atacagtttc aatgaccttg tcattgaccc tgggacgttg
10321 ccattaacat ggtgatcctc agctggcccc attcctatgg cggacctcta aaaacccaac
10381 cctgacccca gcccccagcc atgcccccga ctccctctga ccctgcccaa ggttagcttc
10441 tttatttatt tatttttttt gagacggagt ctcgctgtgt cacccaggct ggagtgcagt
10501 ggtgcaatct cggctcactg caacctctgc ctcccgggtt caagcgattc tcctgcctca
10561 gcctcctgag tagctgggat tataggcaca cgccaccatg cctggctaat ttttgtattt
10621 ttagtagaga tggggtttca ccatgttgac caggctggtc tcaaactcct gaccttgtga
10681 tccgcccacc tcaggctccc aaagtgctgg gattacgggc gtgagccact gtgcccggcc
```

```
10741 caggttagct tctg  agt aaaactgggc tcaacccagg gctg  gat tccagaagcc
10801 gtgctcctaa cccctctgtc ctcagtttag tagggtggct gggaacagtg gtttccctgc
10861 aagctgcaag ggtcagggga cagagcagga tgcggaagtg gcaggtagat aggattcttt
10921 cagcagatat atctaagggc caagatctgt gctgggttct gggcatggag gaaaatcagg
10981 tgtgcatgat ccgtccaagg cctgtgggca aggatggcac aggaacagac atcccatgac
11041 caatgaccta cttgtaacag gtatgaagga agagtggaag gttgcagagg gacccctgct
11101 ttagattggt atgacaggag atccaggaga gcttctagaa tgttctatcc atcactagtc
11161 tctagcccta tgcagctatt taaattttga ttttaattcc ggctgggcac ggtgactcac
11221 gcctgtaatc ccagcccttt gggaggccga gggggggtgg gggtggatca cctgaggtta
11281 ggagttcgag accagcctgg ccaacatggc aaaaccccat ctctactaaa aatacaaaaa
11341 attagcggac gtggtggcag gcacctgtaa tcccagctac tccggaggct gaggcagtag
11401 aattgcttga acctgggacg tagaggttgc agtgagccga gatcaagcca ctgcactcca
11461 acgtgggcga gagaccgaga ctctgcttca aagacaaaac aacaattttt taaaaatttt
11521 aattcaaatg aagtacaatt gcaaatttag cctctgactt gcaccatcct gtatccagtg
11581 ctcaaaagcc agtgtggctg gtggctgcca tattggacag catagatatt gaatacttcc
11641 atcgcctcta gactgaagag atgggagccc aggggcagtg caccgagggg aaggaatagc
11701 taaagcaaag gtctagtagc ctgaaaaaac ttggagaaaa gatggcccct ccatgaggcc
11761 gagtgagagg aaggaagcct tggctgggac cctgccacat ccaatgtcac cggcagatgg
11821 gtacaccccc ttttccccat gcatggattc agctgtccca cagacacatt gactcaggcc
11881 cttggaacta cttcctgtct tgccttagca cgtagacatc acacacatgc atccactcag
11941 gtgggcagtc tcaggccctg ctcccactgc tgtgctcagc gtgcatccag ctcactcaat
12001 agatggtttc tgagcatcga ggtcatgtca gccctggctc taggtctgta ggtgctggac
12061 ctacagcaga aggcaaagac acagactggc aaagacacag cttgtatcca ggttcagggg
12121 tcagggaagg tccctctgtg caagcaaact gtggaacaac gggtggagca ggcccagcaa
12181 gtgcaaaggc ccggaggtgg gaagcgatgc agatatggct ggaggggagg gcggacttca
12241 agggccttgg aggttgggag ccactttcag gctgagcctc ccggcttctc tccccagcat
12301 cccggcggcg caggcgcaga ggagagcgag ctgcaggcct acatcgcaca gtgccaggac
12361 agccccacct ccggcaagtt ccgccgcggg agcggctcgg cctgcagcct tctctgctgc
12421 tgcggaaggt tcgagtcccg ggtctggcac attcagattg gaggttacgg aatggggaaa
12481 ggggagcgag ccagagaaaa ctgacgcccc tcttccctgc ttccttcctc cagggacccc
12541 tcggaggagc attcgctgct ggtgaattga ttcgacctga ctgccgttgg accgtaggcc
12601 ctggactgca gagacccccg cccccgaccc cgcttattta tttgtagggt ttgcttttaa
12661 ggatcggctc cctgtcgcgc ccgaggaggg cctggacctt tcgtgtcgga cccttggggg
12721 cggggagact gggtggggag ggtgttgaat aaaagggaaa ataaatgtgt cgttttcatt
12781 tttagcggga ggagcagtcc ttgcgttaag cggtgtgagg ccctttaagg cgcggccaca
12841 ctcagcatgg cggcctcagt cggccttcca agcatggcgc ggggaggagg ggtgggaggg
12901 tcgggaggga ctgcggtgcg actaggagtg aataatttaa aggggccgcg cctgcggagc
12961 cgggcggaac gctagcggtg ttggcgcgga gtggaccccg gctgcggccc ctgggtgagt
13021 ctgggtttcc gttagcctcg caggggtgtc ccttcgaggg tcgttagcga gcctccgctt
13081 tcccacgatc tgtcctccga ttcttgttaa ctctagactt tctgatgttc ccatacccca
13141 cacgtctcgg caggtgtttc cacaccggta gccagctgtg ccctgaggtg gaagaggacc
13201 ggccacccag gaattttcca agtaacgact cggagtctcc gggattccta tctcccggcc
13261 cccgaatttc
//
```

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Sun, et al.
Title:	METHODS AND COMPOSITIONS FOR THE DETECTION OF MUCOLIPIDOSIS IV MUTATIONS
Appl. No.:	10/754,446
Filing Date:	January 9, 2004
Examiner:	Kapushoc, Stephen Thomas
Art Unit:	1634
Confirmation Number:	7990

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date below.

Marisol Moretto
(Printed Name)

(Signature)

August 10, 2006
(Date of Deposit)

DECLARATION OF WEIMAN SUN, PH.D UNDER 37 C.F.R §1.132

Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Sir:

I, Weiman Sun, Ph.D. hereby declare as follows:

1. I attended the University of California, Davis where I received a Ph.D degree in Pharmacology and Toxicology in 1994.

2. I was a postdoctoral fellow at UCLA Intercampus Medical Genetics Training program for over 3 years. Then, I served as the director of the molecular genetics laboratory (The Genetics Center) and the manager of the molecular pathology lab (UC Irvine) before joining Quest Diagnostics as an associate scientific director and later the R&D manager of the Molecular Genetics Department. I am currently the Scientific Director of Molecular Genetics at Quest Diagnostics Nichols Institute, Assignee of the patent application.

3. In total, I have worked more than 12 years in the field of nucleic acid diagnostic assays. I am the author or co-author of more than 29 published scientific articles and

many abstracts in this field. A brief summary of my accomplishments and a recent copy of my Curriculum Vitae is attached as APPENDIX 1.

4. I am a co-inventor of the above identified patent application. I understand that the Examiner has rejected the claims as being obvious over a combination of prior art references. According to the Examiner, one of ordinary skill would have been motivated to take various features from these references and combine them to obtain the claimed invention and that there would have been a reasonable expectation of success in achieving the combination. The Examiner bases this conclusion on the belief that because the entire sequence of MCOLN1 is known, it would have required nothing more than ordinary skill to develop a MCOLN1 real time assay using any particular primer or probe sequence that can be derived from the MCOLN1 genome. In my opinion, this rationale is flawed. My own experience from 12 years of assay development and from others who have published in this field leads me to conclude that a successful nucleic acid based assay is very much dependent on the particular primers and probe sequences chosen and that there is no way to predict in advance whether particular sequences would be effective.

5. It is my experience that real time amplification assays such as the TaqMan assay are more complex than standard PCR assays because the primers and the probe in a TaqMan format must be able to function together at the same time since the probe must bind to amplified product as it is extended from the primers. The probe in standard PCR is applied after the PCR has been completed and amplicons generated. Because of the more stringent working requirements, primers pairs and probes that might work acceptably in standard PCR are more likely to fail or perform poorly in the TaqMan format. I conclude from this that there would not have been a reasonable expectation of success for combining the probes of Edelmann et al. from standard PCR assays, for use in a TaqMan style PCR assay such as described in Doll et al. as asserted by the Examiner.

6. Unlike mutation analysis methods that analyze amplification products from conventional PCR reactions, our amplification primers as well as mutation detection (allelic-discrimination) probes need to work together in the same homogeneous condition balancing the need for high amplification efficiency and the capability in distinguishing the

7. As described in Section 5.1 (Primer and probe design guidelines) in Real-Time PCR (Advanced Methods Series) (Dorak MT, Oxford: Taylor & Francis, 2006), real-time PCR has different needs than conventional PCR. Different considerations for real-time PCR include, for example, primer melting temperature, probe melting temperature, amplification product length, and placement of primers and probes.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the captioned patent application or any patent issued therefrom.

8. 9. 2006

Date

Weiman Sun, Ph.D.
Quest Diagnostics, Inc.
33608 Ortega Highway
San Juan Capistrano, CA 92675

WEIMIN SUN

23 Cambridge
Irvine, CA 92620
(949) 552-5340
sunw@questdiagnostics.com

SSN: 415-59-0165
Birth Date: June 7, 1965
Marital Status: Married
Citizenship: U.S.A.

EDUCATION

Pharmacology and Toxicology, University of California, Davis. 09/89 - 07/94
Ph.D., September 1994.
Advisor: Dr. Jerold A. Last

Basic Medicine, Shanghai Medical University, 09/82 - 06/88
MD, June 1988

PROFESSIONAL EXPERIENCE

Scientific Director, Molecular Genetics Department, Quest Diagnostics Incorporated, 2002-present

1. Responsible for overall design, performance and maintenance, as well as technical and clinical validation of all testing in Molecular Genetics
2. Manage R&D portfolio design/planning, staffs and projects
3. Review clinical information and laboratory results for various genetic tests to provide final diagnosis and interpretations
4. Provide consultation to physicians and genetic counselors on genetic testing

Research and Development Manager, Molecular Genetics Department, Quest Diagnostics Incorporated, 2001

1. Manage R&D staffs (senior scientists and research associates) and projects
2. Review clinical information and laboratory results for various genetic tests to provide final diagnosis and interpretations
3. Provide consultation to physicians and genetic counselors on genetic testing
4. Participate in QA&QC programs

Associate Scientific Director of Molecular Genetics Department, Quest Diagnostics Incorporated, 2000

1. Review clinical information and laboratory results for various genetic tests to provide final diagnosis and interpretations
2. Provide consultation to physicians and genetic counselors on genetic testing
3. Manage R&D staffs and projects
4. Participate in QA&QC programs

Manager of Diagnostic Molecular Pathology Laboratory, UC Irvine, 1999 - 2000

1. Set up the molecular diagnostics laboratory, including purchasing equipment and supplies, designing laboratory layout and developing/implementing quality control program
2. Developed and validated molecular diagnostic assays, including thrombophilia variant analysis (Factor V Leiden, prothrombin and MTHFR), Fragile X syndrome, HFE genotype analysis (C282Y and H63D) and parentage/genetic identity testing
3. Managed the laboratory according to CLIA/CAP guideline, troubleshot technical problems and trained technologist and participated in proficiency/interlab exchange testing
4. Written standard operational procedures of the laboratory and pamphlets for test information
5. Designed and developed a PC database for general laboratory management

Director of Molecular Genetics Laboratory, Genetics Center, 1998

1. Set up the molecular diagnostics laboratory, including purchasing equipment and supplies, designing laboratory layout, developing quality assurance protocols and training technologists.

2. Developed and validated molecular diagnostic assays, including cystic fibrosis (analysis for 33 mutations and 1 polymorphism), Fragile X syndrome, Ashkenazi Jewish genetic diseases panel (Canavan, CF, Gaucher and Tay-Sachs), genetic identity tests, myotonic dystrophy, BCR-ABL translocation (RNA-based), Prader-Willi/Angelman syndromes (methylation PCR) and sex chromosome analysis.
3. Supervised daily laboratory operation, troubleshot technical problems, reviewed and signed-out diagnostic cases.
4. Developed report forms on molecular genetic tests; given presentations and lectures to clinicians and written newsletter articles on molecular tests.

Postdoctoral Work, UCLA, 1994 – 1997

1. Identified the molecular defect in aryl hydrocarbon receptor protein expressed by a mutant strain of a mouse hepatoma cell line. Characterized the effect of a single point mutation in the coding region of the mutant protein on its functionality in protein (ARNT)-heterodimerization, DNA binding and transactivation.
2. Studied possible human genetic polymorphisms in inducibility of cytochrome *P-450IA1* (*CypIA1*) gene by using peripheral blood mononuclear cells collected from healthy volunteers and placenta tissue. Developed competitive rt-PCR assay to accurately quantify the expression of *CYP1A1* gene as well as its regulators. Developed methodology to amplify and clone full-length coding cDNA of the relevant genes for *in vitro* expression and functional analysis.
3. Applied representational difference analysis (RDA) technique to study the tumor-promotion activity of TCDD by isolating and analyzing the affected genes.
4. Received training in applying DNA technology to clinical diagnostics. Developed a simple PCR-based procedure to test for Familial Haemochromatosis and a RNA-based assay to detect chromosome translocation.

Graduate Study, UC Davis, 1989 - 1994

1. Studied the acute effects of airborne toxicants (ozone and tobacco smoke) on human airway epithelial cells *in vitro*. Defined cytotoxicities of air pollutants with various viability assays. Analyzed changes in protein synthesis patterns in injured cells. Characterized the induced expression of a 45kDa protein by ozone and environmental tobacco smoke (ETS). Generated and purified internal peptides from the 45kDa protein for N-terminal micro-sequencing analysis.
2. Tested the importance of inflammation in enhancing the direct toxic effects of ozone and nitrogen dioxide mixture to rat lungs *in vivo*.
3. Teaching Assistant (1992) for Department of Environmental Toxicology in *Air Pollution and Inhalation Toxicology*. Participated in designing and grading homework and exam questions, helped students during office hours, coordinated guest lecturers and provided demonstration facilities.

Research Assistant, Shanghai Medical University, 1982 - 1988

Used immunohistochemistry methods to study the distribution and expression of desmin in human fetal organs and tissues, as well as poorly differentiated sarcomas; Professor Yue-er Zhang, Department of Pathology

PUBLICATIONS

Last, J.A., Wu, R., Jin, C., Gelzleichter, T., **Sun, W.**, Armstrong, L.C. (1990) Particle-cell interactions – lung fibrogenesis. Journal of Aerosol Medicine 3(S1): S61-S74

Sun, W., Wu, R., and Last, J.A. (1994) Coordinated Expression of 45 kDa Protein Synthesis and Ozone Toxicity in a Human Bronchial Epithelial Cell Line. American Journal of Respiratory Cell and Molecular Biology 10: 673-82

Sun, W. (1994) Cytotoxicity of Air Pollutants in Airway Epithelium in vitro.
(Ph.D thesis)

Last, J.A., **Sun, W.**, and Witschi, H. (1994) Ozone, NO, and NO_2: Oxidant Air Pollutants and More. Environmental Health Perspective 102: 179-84

Tarkinton, B.K., Wu, R., **Sun, W.**, Nikula, K.J, Wilson, D.W., and Last, J.A. (1994) In Vitro Exposure of Tracheobronchial Epithelial Cells and of Tracheal Explants to Ozone. Toxicology 88: 51-68

Sun, W., Wu, R., and Last, J.A. (1995) Effects of exposure to environmental tobacco smoke on a human tracheobronchial epithelial cell line. Toxicology 100: 163-74

Hankinson, O., Bacsi, S.G., Fukunaga, B.N., Kozak, K.R., McNulty, S.E., Minehart, E., Probst, M.R., Reisz-Porszasz, S., **Sun, W.**, and Zhang, J. (1995) Genetic Analysis of the Aryl Hydrocarbon Receptor Nuclear Translocator Protein (ARNT). European Journal of Drug Metabolism and Pharmacokinetics Special Issue: 31-2

Sun, W., Zhang, J., and Hankinson, O. (1997) A mutation in the aryl hydrocarbon receptor (AHR) in a cultured mammalian cell line identifies a novel region of AHR that affects DNA binding. Journal of Biological Chemistry 272(50): 31845-54

Antilla, S., Lei, X., Elovaara, E., Karjalainen, A., **Sun, W.**, Vainio, H., and Hankinson, O. (2000) An uncommon phenotype of poor inducilbility of CYP1A1 in human lung is not ascribable to polymorphisms in the *AHR*, *ARNT*, or *CYP1A1* genes. Pharmacogenetics 10: 1-11

Roth MD, Marques-Magallanes JA, Yuan M, **Sun W**, Tashkin DP, Hankinson O. (2001) Induction and Regulation of the Carcinogen-Metabolizing Enzume, CYP1A1, by Marijuana Smoke and Δ^9-Tetrahydrocannabinol. American Journal of Respiratory Cell and Molecular Biology. 24(3):339-44.

Strom, CS., Huang, S., Buller, A., Redman, J., Crossley, B., Anderson, B., Entwistle, T., and **Sun, W.** (2002) Cystic Fibrosis Screening Using the College Panel: Platform Comparison and lessons from the first 20,000 samples. Genetics in Medicine 4(4): 289-96

Strom, CS, Huang, S, Chen, C, Buller, A, Peng, M, Quan, F, Redman, R, **Sun, W.** (2003) Extensive sequencing of the Cystic Fibrosis Transmembrane Regulator gene: Assay validation and unexpected benefits of developing a comprehensive test. Genetics in Medicine, 5(1): 9-14

French C, Li C, Strom C, **Sun W**, Van Atta R, Gonzalez B, Wood M. (2004) Detection of the factor V Leiden mutation by a modified photo-cross-linking oligonucleotide hybridization assay. Clinical Chemistry 50(2):296-305. Epub 2003 Dec 04

Strom CM, Clark DD, Hantash FM, Rea L, Anderson B, Maul D, Huang D, Traul D, Chen Tubman C, Garcia R, Hess PP, Wang H, Crossley B, Woodruff E, Chen R, Killeen M, **Sun W**, Beer J, Avens H, Polisky B, Jenison RD. (2004) Direct visualization of cystic fibrosis transmembrane regulator mutations in the clinical laboratory setting. Clinical Chemistry, 50(5):836-45. Epub 2004 Mar 09.

Strom CM, Crossley B, Redman JB, Buller A, Quan F, Peng M, McGinnis M, **Sun W.** (2004) Cystic fibrosis screening: lessons learned from the first 320,000 patients. Genetics in Medicine. 6(3):136-40

Strom CM, Crossley B, Redman JB, Quan F, Buller A, McGinniss MJ, **Sun W.** (2004) Molecular screening for diseases frequent in Ashkenazi Jews: lessons learned from more than 100,000 tests performed in a commercial laboratory. Genetics in Medicine. 6(3):145-52.

Monaghan KG, Highsmith WE, Amos J, Pratt VM, Roa B, Friez M, Pike-Buchanan LL, Buyse IM, Redman JB, Strom CM, Young AL, **Sun W.** (2004) Genotype-phenotype correlation and frequency of the 3199del6 cystic fibrosis mutation among I148T carriers: results from a collaborative study. Genetics in Medicine, 6(5):421-5.

Huang D, Chen C, **Sun W**, Strom CM, Bender RA. (2004) High-throughput gene sequencing assay development for hereditary nonpolyposis colon cancer. Clinical Colorectal Cancer, 4(4):275-9

Ahmed SA, Bailey-Snow K, Highsmith WE, **Sun W**, Fenwick RG, and Mao R (2005) Nine novel germline gene variants in the RET proto-oncogene identified in twelve unrelated cases. J Mol Diagn. 7(2):283-8.

Brown NM, Pratt VM, Buller A, Pike-Buchanan L, Redman JB, **Sun W**, Chen R, Crossley B, McGinniss MJ, Quan F, and Strom CM. (2005) Detection of 677CT/1298AC "double variant" chromosomes: implications for interpretation of MTHFR genotyping results. Genetics in Medicine, 7(4):278-82

McGinniss MM, Chen C, Redman JB, Buller A, Quan F, Peng M, Giusti R, Hantash F, Huang D, **Sun, W,** and Strom C (2005) Extensive Sequencing of the CFTR gene: lessons learned form the first 157 patient samples. Human Genetics, 118: 331-8

Hantash F, Anderson B, Redman J, Buller A, McGinniss MM, Peng M, Quan F, **Sun W**, and Strom C. (2006) Novel and Recurrent Rearrangements in the CFTR Gene: Clinical and Laboratory Implications for Cystic Fibrosis Screening. Human Genetics, 119: 126-36

Hantash, F, Milunsky A, Wang Z, Anderson B, **Sun W**, Anguino A, and Strom C (2005) CFTR Gene Deletions and Duplications in Men with Congenital Bilateral Absence of Vas Deferens. Genetics in Medicine (GIM-D-05-00074, in press)

Strom CM, Janeczko RA, Anderson B, Redman J, Quan F, Buller A, McGinniss MJ, **Sun W** (2005) Technical validation of a multiplex platform to detect thirty mutations in eight genetic diseases prevalent in individuals of Ashkenazi Jewish descent. Genetics in Medicine, 7(9):633-9.

Huang D, **Sun W**, Strom CM. (2005) Sequence variations in AGTR2 are unlikely to be associated with X-linked mental retardation. American Journal of Medical Genetics. A139(3):243-4.

Hantash FM, Olson SC, Anderson B, Buller A, Chen R, Crossly B, **Sun W**, Strom CM. (2006) Rapid one-step carrier detection assay of mucolipidosis IV mutations in the Ashkenazi Jewish population. Journal of Molecular Diagnostics. 8(2):282-7.

Hantash FM, Milunsky A, Wang Z, Anderson B, **Sun W**, Anguiano A, Strom CM. (2006) A large deletion in the CFTR gene in CBAVD. Genetics in Medicine, 8(2):93-5.

Sun W, Anderson B, Redman J, Milunsky A, Buller A, McGinniss MM, Quan F, Anguino A, Huang S, Hantash F and Strom C. (2006) The CFTR 5T Variant Has a Low Penetrance in Females That is Partially Attributable to Its Haplotype. Genetics in Medicine, 8:339-45

Strom CM, Janeszco R, Quan F, Wang SB, Buller A, McGinniss M, **Sun W.** (2006) Technical validation of a tm biosciences luminex-based multiplex assay for detecting the american college of medical genetics recommended cystic fibrosis mutation panel. Journal of Molecular Diagnostics. 8(3):371-5.

ABSTACTS AND MEETING PRESENTATIONS

Sun, W., Chau, D., Krolewski, J., Selsted, M., Kagan, R., and Fenwick, R. (2000) A multiplex allele-specific amplification assay detects two common variants in the human HFE gene associated with hereditary hemochromatosis. (presented at the AMP annual meeting)

Sun, W., Buller, A, Huynh, N., Huang, S., Vu, M., Avila, L., Chen, C., Bower, B., York, S., Entwistle, T., and Strom, C. (2001) Genotype frequencies of C282Y and H63D alleles in the hemochromatosis (HFE) gene from patient samples submitted to a reference laboratory for HFE genotyping. (presented at the 2001 ASHG

annual meeting)

Anderson, B., **Sun, W.**, Buller, A., Huang, S., Hantash, F., Wang, S., Entwistle, T., Strom, CS. (2001) A novel technique to accurately assess engraftment percentages. (presented at 2002 ACMG annual meeting)

Qu, K., **Sun, W.,** Huynh, N., Strom, C., Popov, J., and Sferruzza, A. (2002) Simultaneous Detection of Methylenetetrahydrofolate Reductase Mutations C677T and A1298C by Fluorescent Restriction Fragment Length Polymorphism. (Presented at the 2002 AACC annual meeting)

Sun W, Buller A, Fenwick R, Hantash F, Huang S, Peng M, Redman J, and Strom CS (2002) Spectrum of Mutations Detected in the RET Proto-oncogene Associated with Multiple Endocrine Neoplasia Type 2 (Presented at 2002 ASHG annual meeting)

Strom C, **Sun W**, Zoleikhaeian M, Fenwick R, York M, Entwistle T. (2002) Comparison of SNP Detection Methods and Instrumentation Platforms (Presented at 2002 ASHG annual meeting)

Sun W, Redman J, Wallenstein R, McCarrier J, Lee D, Buller A, McGinniss M, Quan F, Huang S, and Strom C. (2003) Comprehensive Sequence Analysis Ruled out Disease Association with the CFTR Gene in a Family with Atypical CF Presentations (Presented at 2003 ASHG annual meeting)

Anderson B, **Sun W**, Redman J, Buller A, McGinniss M, Quan F, and Strom C (2004) CFTR Gene Haplotype Determines the Phenotype Associated with the 5T Variant (Presented at 2004 ASHG annual meeting)

Sun W, Potts S, Peng M, Buller A, McGinniss M, Quan F, Taylor J, and Strom C (2005) Novel Mutations and Variants Identified in the MECP2 Gene (Presented at 2005 ASHG annual meeting)

(Only presentations with my being a 1st or 2nd author are included)

PROFESSIONAL BOARD CERTIFICATION

American Board of Medical Genetics (Clinical Molecular Genetics,)
New York State Certification of Qualification for Laboratory Director (DNA)
Clinical Genetic Molecular Biologist by the State of California
Clinical Genetic Molecular Biologist Scientist by the State of California
Clinical Laboratory Scientist (Molecular Biology) by National Certification Agency (NCA)

HONORS

- Regents Fellowship, UCDavis, 1990-1991
- Nonresident Tuition Fellowship, UCDavis, 1989, 1991-1993
- GSA Travel Grant, UCDavis, 1993
- Research Training Grant Fellowship, American Lung Association, 1995-1996
- Intercampus Medical Genetics Training Grant, NIH, 1996 (Jan.) – 1997 (Dec.)

PROFESSIONAL AFFILIATIONS

American Society of Human Genetics (ASHG)
Association for Molecular Pathology (AMP)
American Association for the Advancement of Science (AAAS)
Phi Sigma Biological Honor Society (elected and declined)

REFERENCES

Charles M. Strom, MD, Ph.D. 949-728-4701
Genetic Testing Center, Quest Diagnostics, San Juan Capistrano, CA 92690

Michael E. Selsted, MD, Ph.D. 949-824-2350
Department of Pathology, UC Irvine, CA 92697
Oliver Hankinson, Ph.D. 310-825-2936
Department of Pathology and Laboratory Medicine, UCLA, CA 90095
Wayne W. Grody, MD, Ph.D. 310-825-5648
Department of Pathology and Laboratory Medicine, UCLA, CA 90095
Jerold A. Last, Ph.D. 530-752-6230
Department of Internal Medicine, University of California, Davis, CA 95616
Yue-er Zhang, MD.
Department of Pathology, Shanghai Medical University, Shanghai, China 200032